BIOSYNTHESIS AND CYTOTOXICITY OF KOJIC ACID FROM *ASPERGILLUS NIGER*

Dissertation

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The Tamil Nadu DR.M.G.R. Medical University, Chennai

In partial fulfillment for the award of the degree of

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In

PHARMACEUTICAL BIOTECHNOLOGY

By

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APRIL - 2013

DECLARATION

I hereby declare that this thesis work entitled **"BIOSYNTHESIS AND CYTOTOXICITY OF KOJIC ACID FROM *ASPERGILLUS NIGER* "** submitted to the Tamil Nadu Dr.M.G.R. Medical University, Chennai was carried out by me in the Department of Pharmaceutical Biotechnology, Ultra College of Pharmacy, Madurai under the valuable and efficient guidance of **Mr. K. Natarajan, M. Pharm.,** Assistant Professor, Department of Pharmaceutical Biotechnology, Ultra College of Pharmacy, Madurai during the academic year May 2012- April 2013. I also declare that the matter embodied in it is a genuine work and the same has not formed the basis for the award of any degree, diploma, associate ship, fellowship of any other university or institution.

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Dedicated To

Mother,

Father,

Brother's & Guide

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1. INTRODUCTION

1.1 Aspergillus species

Aspergillus is a genus of around 200 molds found throughout much of nature worldwide. Aspergillus was first catalogued in 1729 by the Italian priest and biologist pier Antonio micheli. Viewing the fungi under microscope, Micheli was reminded of the shape of an *aspergillum* (holy water sprinkler) and named the genus accordingly (Taylor R. T. Dagenais et al *2009).*

Aspergillus species are highly aerobic and are found in almost all oxygen-rich environments, where they commonly grow as molds on the surface of a substrate, as a result of the high oxygen tension. "In recent studies, increased levels of Reactive oxygen Species (ROS) were shown to be correlated with increased levels of aflatoxins biosynthesis in *Aspergillus parasiticus* (Robert B Kammer et al 1974*)*. Commonly fungi grow on carbon- rich substrates such as monosaccharides (such as glucose) and polysaccharides (such as amylase). *Aspergillus* species are common contaminants of starchy foods (such as bread and potatoes), and grow in or many plants and trees.

In addition to growth on carbon sources, many species of *Aspergillus* demonstrate oligotrophy where they are capable of growing in nutrient-depleted environments, or environments in which there is a complete lack of key nutrients. *Aspergillus niger* is a prime example of this; it can be found growing on damp walls, as a major component of mildew. Species of *Aspergillus* are important medically and commercially. Some species found in animals have been studied for years. Some species found in animals have been described as new and specific to the investigated disease and others have been known as names already in use for organisms such as saprophytes. More than 60 names of species of *Aspergillus* are encountered in the literature of pathology. For humans cases a whole range of infections such as infection to the external ear, skin lesions and ulcers classed as mycetomas. Out of the approximately 200 species of *Aspergillus* there are about 16 which can cause infection in humans. These include:

- *Aspergillus clavatus*
- *Aspergillus flavus*
- *Aspergillus fumigatus*
- *Aspergillus glaucus*

- *Aspergillus nidulans*
- *Aspergillus niger*
- *Aspergillus oryzae*
- *Aspergillus terreus*
- *Aspergillus ustus*
- *Aspergillus versicolor*

Aspergillus Symptoms

Aspergillus spores are everywhere and although we constantly breathe *aspergillus* spores in, it is usually not in high enough quantities to affect our health. However if a person is breathing in a much higher amount of *Aspergillus* spores than normal, as can happen if *Aspergillus* is growing in a person's home, then they can suffer negative health symptoms. *If Aspergillus* exposure increases beyond a person's natural tolerance levels there are three main types of health effects they can suffer:

- Allergic symptoms: People who are allergic to *Aspergillus* spores will suffer allergic reactions if they are exposed to high enough levels of *Aspergillus* spores. People with severe asthma often are sensitive to *Aspergillus* and can suffer asthma attacks from exposure to *Aspergillus* spores.
- Toxic symptoms:These are caused by the mycotoxins (such as aflatoxin, a very potent carcinogen) produced by some species of *Aspergillus*,especially *Aspergillus flavus.*
- Infection: *Aspergillus* species can infect humans (also animals) and begin to grow inside them, especially in the lungs. Usually only people with weakened immune systems will be susceptible to infection by *Aspergillus* infections are categorized in the group of diseases called Aspergillosis,(Walsh, TJ et al.2008*).*

Aspergillus Mycotoxins

Some species of *Aspergillus* molds can produce mycotoxins. These *Aspergillus* species do not constantly produce mycotoxins but can be triggered to by factors such as stress. These mycotoxins are mostly produced by the species *Aspergillus flavus*. The mycotoxins most produced by these *Aspergillus* molds are aflatoxins.

As well as being very toxic,aflatoxins are highly carcinogenic (cancer causing) especially in animals and they have also resulted in miscarriage in animals

such as sheep and cows. Crops and foods such as peanuts and maize are often contaminated by *Aspergillus* leading to mycotoxins being present. There are safety limits set for the amount of aflatoxins allowed in food. However there are no similar levels set for aflatoxin concentration in the air of buildings and there has not been enough.

Types of aspergillosis

The group of diseases in humans which are caused by *Aspergillus* exposure is known as Aspergillosis (Rosa C et al., 2011,1467-71.). The main diseases of Aspergillosis are:

- Allergic broncho pulmonary aspergillosis
- Acute invasive aspergillosis

Allergic Bronchopulmonary Aspergillosis

Allergic bronchopulmonary aspergillosis (ABPA) is a diseases where a person's immune system is hypersensitive to *Aspergillus* spores. This hypersensitivity causes allergic reaction in the person when they are exposed *to Aspergillus* as the immune system tries to expel the spores from the body. People with cystic fibrosis or asthma are especially vulnerable to Allergic bronchpulmonary aspergillosis, with approximately 5% of asthmatics suffering this disease at some point in their life. Allergic bronchpulmonary aspergillosis is usually caused by *Aspergillus fumigatus*. Symptoms:

- Difficulty breathing, shortness of breath and breathlessness
- Wheezing
- Symptoms of asthma
- Coughing up mucus
- Coughing blood
- Sinusitis(infection or inflammation of the sinuses)
- Loss of appetite
- Fever
- General malaise and feeling unwell
- In later stages can cause lung damage(fibrosis)

Tests for allergic bronchopulmonary aspergillosis can be performed through x-rays, skin tests or blood tests. The treatment for allergic bronchopulmonary aspergillosis is for steroids to be taken through mouth or nasal spray. An antifungal drug called itraconazole can also help to treat the disease in conjunction with steroids.

Chronic Pulmonary Aspergillosis and Aspergillomas

This disease is caused by *Aspergillus* infecting the body and growing in cavities in the lungs. These cavities in the lungs would usually have to be created by a previous health problem such as tuberculosis. Once the *Aspergillus* mold has infected the lungs it begins to grow into a fungal ball (called an aspergilloma or mycetoma) which then makes the person sick because of the allergens or toxins it puts out into the person's body. At first the symptoms of an aspergilloma might not be noticeable but as time goes on it can show in health problems such as:

- Breathing problems
- Chronic coughing
- Coughing up blood
- Losing weight
- Tiredness
- Fatigue

1.2 *Aspergillus niger*

Aspergillus niger is a fungus and one of the most common species of the genus *Aspergillus*. It causes a disease called black mold on certain fruits and vegetables such as grapes, onions. Peanuts are a common contaminant of food. It is black colonies can be confused with those of stachybotrys (species of which have also been called "black mold"). Some strains of *Aspergillus niger* have been reported to produce potent mycotoxins called ochratoxins, but other sources disagree,claiming the later report is based upon misidentification of the fungal species. Genomic sequences comparisons have also led to the demonstration of another Fumonisins another important mycotomicin. Recent evidence suggests some true *Aspergillus niger* strains do produce ochratoxin A (Abarca M et al 1994). *Aspergillus niger* is included in *Aspergillu*s subgenus circumdati, section nigri. The section nigri includes 15 related black spored species that may be confused with *Aspergillus niger,* including

Aspergillus tubingensis, Aspergillus foetidus Aspergillus carbonarins and *Aspergillus awamori.*

Figure 1: ***Aspergillus niger*** **grown on Sabouraud agar medium. 100 x magnification**

A number of morphologically similar species were recently described by Samson et al 2001. *Aspergillus niger* causes black mold of onions. Infection of onion seedlings by *Aspergillus niger* can become systemic, manifesting only when conditions are conductive. *Aspergillus niger* causes a common post harvest disease of onions, in which the black conidia can be observed between the scales of the bulb. The fungus also causes disease in peanuts and in grapes. *Aspergillus niger* is less likely to cause human disease than some other *Aspergillus* species, but if large amounts of spores are inhaled, a serious lung disease, aspergillosis can occur. Aspergillosis is particularly frequent among horticultural workers who inhale peat dust, which can be rich in Aspergillus spores. Less commonly, it has been found on the walls of ancient Egyptian tombs and can be inhaled when the area is disturbed. *Aspergillus niger* is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss and in severe cases, damage to the ear canal and tympanic membrane. The black aspergilli are some of the most important mycotoxigenic food and feed contaminants, especially in postharvest decay of fresh and dried fruits and certain vegetables, nuts, beans, and cereals. This is due to their fast growth, pH tolerance, and high abudance in many environments. For the analytical chemist, issues such as fungal taxonomy and correct identification may seem of low relevance, but in fact biosystematics is a vital part of mycotoxin research

and food safety. Since the profile of mycotoxins and other secondary metabolites is species specific, correct identification at the species level provides the key for planning the analytical determination of all relevant compounds. The *Aspergillus niger* group (the black *Aspergilli, Aspergillus* subgenus Circumdati section Nigri) comprises 18 species, of which *A. niger, A.tubingensis, A.brasiliensis, A. acidus, A. carbonarius,* and *A.ibericus* are common, whereas the remaining species are rare and found mainly in tropical regions. A clarification of *Aspergillus* section nigri using the B-tubulin and calmodulin geners showed that three clades could be distinguished. The *A. niger* clade, a clade consisting of the two rare species *A. homomorphus* and *A. ellipticus*, and the clade of uniseriate black Aspergilli (*A.aculeatinus. A. aculeatus, A.japonicus,* and *A.uvarum*), the members of which differ significantly from the remaining black aspergilli regarding their morphology, physiological behavior, and secondary metabolite production (e.g., producers of neoxaline, asperparalines, secalonic acids, asperamide, and aculeasins). The identity and metabolite production of the uniseriate black *aspergilli* is usually not confused with the identity and metabolite production *of A.niger* and other biseriate black *Aspergilli.*

A.niger and A.tubingensis are probably the most common of the black *Aspergill*i (Samson RA, et al 2001). Secondary metabolites from these *Aspergilli*, the producing organism has been identified as a black *Aspergillus* and then in many cases incorrectly named *A.niger.* A wrong identification may be further complicated by insufficient molecular identification based on sequencing of ribosomal DNA with low resolution. Apolyphasis approach where many different types of characters (microscopy, metabolite profiling, molecular methods) are used is recommended for the identification of these *aspergilli* Certain molecular methods have proven quite successful, including restriction fragment length polymorphism and B-tubulin or calmodulin sequencing. Unit recently, the main mycotoxin from the black *aspergilli* was considered to be ochratoxin A (OTA), produced in variable amounts within certain species of the group. *A. carbonarius* consistently produces large amounts of OTA, whereas only 6-10% of members of the *A.niger* group produce OTA and in 10-1000 lower amounts. The third species producing OtA in section Nigri is *A. sclerotio niger*, but this species has only been found once in coffee. However, *A.niger* clearly has the widest distribution and has been reported to grow and damage a much larger number of crops and foods worldwide, including corn, peanuts, raisins, onions,

mango, apples, and dried meat products. This combined with the recent discovery that *A. niger* can also produce fumonisin B2 (FB2) and fumonisin B4 (FB4) necessitates the addition of fumonisins in a number of food and feed screening programs. *A.niger* exhibits a remarkably versatile metabolism, which has made it one of the most important production organisms used for industrial fermentations. Since 1923, *A.niger* has been exploited commercially for its production of citric acid, mostly for use in food, cosmetics, and pharmaceutical preparations. In addition, the fungus has been a rich source of industrial enzymes such as a-amylases, cellulases, and pectinases for use in the food industries since the 1960. *A.niger* possesses posttranslational mechanisms capable of correctly processing proteins that are difficult to express in traditional host organisms. As a result, it is widely used as a cell factory for heterologous expression of proteins. *A.niger* has been considered to be nontoxic under industrial conditions, and thus to be considered a safe production organism. As a result, quite a number of *A.nigr* fermentations have been granted the generally regarded as safe (GRAS) status by the US Food and Drug Administration. However, the potential presence of both OTA and fumonisins in *A. niger* emphasizes the need to adjust and/or reconsider the screening prodcedures for simultaneous targeting of multiple classes of mycotoxins. Important secondary metabolites produced by members of the *A.niger* group relevant to the food, feed, and biotechnology industries were also studied. Critically scrutinizing the existing literature for reports of secondary metabolites claimed to be produced by *A.niger,* however often just found in a single strain or on a single occasion. Analytical results from 25 years of metabolite profiling at the Center for Microbial Biotechnology of the black *Aspergilli* (were presented already) . The strains investigated come from a large in-house collection (IBT Collection) and other fungal collections.

Industrial uses

A.niger is cultured for the industrial production of many substances. Various strains of *A.niger* are used in the industrial preparation of citric acid (E330) and gluconic acid (E574) and have been assessed as acceptable for daily intake by the World Health Organisation, *(*Laboni Majumder et al 2010). *A.niger* fermentation is generally recognized as safe" (GRAS) by the United States Food and Drug Administration under the Federal Food, Drug, and Cosmetic Act. Many useful enzymes are produced using industrial fermentation of *A.niger.* For example*, A niger*

glucoamylase is ued in the production of high fructose corn syrup, and pectinases are used in cider and wine clarification. Alpha-galactosidase, an enzyme that breaks down certain complex sugars, is a component of Beano and other products that decrease flatulence. Another use of *A.niger* within the biotechnology industry is in the production of magnetic isotope-containing variants of biological macromolecules of NMR analysis.

Other Uses

A.niger is the main agent in the fermentation of Pu-erhtea, the Okinawan awamori spirit, and some varieties of shochu. It was reported that a secreted RNase (J. Holzmann and P. Frank,et al 2008) produced by *A.niger* called actibind has antiangiogenic and anticarcinogenic characteristics. *A.niger* is also cultured for the extraction of the enzymes glucose oxidase (GO) and Alpha-galactosidase (AGS). Glucose oxidase is used in the design of glucose biosensors, due to its high affinity for B-D-glucose. Alpha-galactosidase can be produced by *A.niger* fermentation; it is used to hydrolyze alpha 1-6 bonds found in melibiose, raffinose, and stachyose.

1.3 Mycotoxins

Mycotoxins are toxic, secondary metabolites of low molecular weight produced by naturally occurring fungi, they are capable of causing disease and death in humans and other animals (Chu 1992) . Because of their pharmacological activity, some mycotoxins or mycoztoxin derivatives have found use as antibiotics, growth promotants, and other kinds of drugs.

The term mycotoxin was coined in 1962 in the after math of an unusual veterinary crisis near London, England, during which approximately 100,000 turkey poults died (Bennett JW and Klch M 2003 16(3):497-516) . When this mysterious turkey X disease was linked to a peanut (groundnut) meal contaminated with secondary metabolites from *Aspergillus flavus* (aflatoxins), it sensitized scientists to the possibility that other occult mold metabolites might be deadly. Soon, the mycotoxin rubric was extended to include a number of previously known fungal toxins (e.g., the ergot alkaloids), some compounds that had originally been isolated as antibiotics (e.g., patulin), and a number of new secondary metabolites revealed in screens targeted at mycotoxin discovery (e.g., ochratoxin A).

Toxins are especially produced under certain conditions including the following :

- Host plant stress in the field: poor soil fertility, insect damage, high/low temperature or moisture.
- Harvesting: late harvesting, dry crops, slow storage filling eg silage clamp, soil contamination.
- Storage: wet grain, poor silage packing, incorrect fermentation of ensiled products.
- Finished feeds and forages: poor hygiene, exposure to air/moisture, incorrect storage (temperature/moisture).

Most fungi are aerobic (use oxygen) and are found almost everywhere in extremely small quantities due to the minute size of their spores. They consume organic matter wherever humidity and temperature are sufficient. Where conditions are right, fungi proliferate into colonies and mycotoxin levels become high. The reason for the production of mycotoxins is not yet known; they are necessary for neither growth nor the development of the fungi. Because mycotoxins weaken the receiving host, the fungus may use them as a strategy to better the environment for further fungal proliferation. The production of toxins depends on the surrounding intrinsic and extrinsic environments and the toxins vary greatly in their severity, depending on the organism infected and its susceptibility, metabolism, and defense mechanisms. Some of the health effects found in animals and humans include death, identifiable diseases or health problems, weakened immune systems without specificity to a toxin, and as allergens or irritants. Some mycotoxins are harmful to other micro-organisms such as other fungi or even bacteria; penicillin is one example.It has been suggested that mycotoxins in stored animal feed are the cause of apparent sex change in hens (Global mycotoxin information Resource).

Mycotoxins can appear in the food chain as a result of fungal infection of crops, either by being eaten directly by humans or by being used as livestock feed. Mycotoxins greatly resist decomposition or being broken down in digestion, so they remain in the food chain in meat and dairy products. Even temperature treatments, such as cooking and freezing, do not destroy some mycotoxins. Although various wild mushrooms contain an assortment of poisons that

are definitely fungal metabolites causing noteworthy health problems for humans, they are rather arbitrarily excluded from discussions of mycotoxicology. In such cases the distinction is based on the size of the producing fungus and human intention. Mycotoxin exposure is almost always accidental whereas with mushrooms improper identification and ingestion causing mushroom poisoning is commonly the case. Ingestion of misidentified mushrooms containi classification schemes tend to reflect the training of the person doing the categorizing. Clinicians often arrange them by the organ they affect. Thus, mycotoxins can be classified as hepatotoxins, nephrotoxins, neurotoxins, immunotoxins, and so forth. Cell biologists put them into generic groups such as teratogens, mutagens, carcinogens, and allergens. Organic chemists have attempted to classify them by their chemical structures (e.g.,lactones, coumarins); biochemists according to their biosynthetic origins (polyketides, amino acid-derived, etc.); physicians by the illnesses they cause (e.g., St. Anthony's fire, stachybotryotoxicosis). And mycologists by the fungi speacies that produce theme.g., *Aspergillus* toxins, *Penicillium* toxins). The mycotoxins may result in hallucinations.

Table 1: Some mycotoxins, their sources and potential toxicities

Toxins	**Producing fungi**	**Toxicities**
Aflatoxin	*Aspergillus flavus*	Hepatocarcinogen
Ochratoxins	*Aspergillus ochraceus*	Hepatotoxin
Patulin	*Penicilliumc expansum*	Brain &lung hemorrhage
Sterigmatocystin	*Aspergillus flavus*	Hepatocarcinogen
Vomitoxin (Deoxynivalenol)	*Fusarium graminearum*	Vomiting
Rubratoxin	*Penicillium rubrum*	Liver hemmorrhage and fatty infiltration
Rugulosin	*Penicillium islandicum*	Nephrosis & liver damage

Mycotoxicoses are the animal diseases caused by mycotoxins; mycotoxicology is the study of mycotoxins . While all mycotoxins are of fungal origin, not all toxic compounds produced by fungi are called mycotoxins. The target and the concentration of the metabolite are both important. Fungal products that are mainly toxic to bacteria (such as penicillin) are usually called antibiotics. Fungal products that are toxic to plants are called phytotoxins. The distinction between a mycotoxin and a mushroom poison is based not only on the size of the producing

fungus, but also on human intention. Mycotoxin exposure is almost always accidental. In contrast, with the exception of the victims of a few mycologically accomplished murderers, mushroom poisons are usually ingested by amateur mushroom hunters who have collected, cooked, and eaten what was misidentified as a delectable species (J.W.Bennett and M.Klch 2003).

Mycotoxicosis is the term used for poisoning associated with exposures to mycotoxins. The symptoms of a mycotoxicosis depend on the type of mycotoxin; the concentration and length of exposure; as well as age, health, and sex of the exposed individual. The synergistic effects associated with several other factors such as genetics, diet, and interactions with other toxins have been poorly studied. Therefore it is possible that vitamin deficiency, caloric deprivation, alcohol abuse, and infectious disease status can all have compounded effects with mycotoxins.In turn, mycotoxins have the potential for both acute and chronic health effects via ingestion, skin contact, and inhalation. These toxins can enter the blood stream and lymphatic system, they inhibit protein synthesis, damage macrophage systems, inhibit particle clearance of the lung, and increase sensitivity to bacterial endotoxin.

In 2004 in Kenya, 125 people died and nearly 200 others were treated after eating aflatoxin-contaminated maize. The deaths were mainly associated with homegrown maize that had not been treated with fungicides or properly dried before storage. Due to food shortages at the time, farmers may have been harvesting maize earlier than normal to prevent thefts from their fields, so that the grain had not fully matured and was more susceptible to infection.

STRATEGIES FOR PREVENTION AND CONTROL OF MYCOTOXINS

In general, prevention of the contamination of fungi and their mycotoxins in agricultural commodities can be divided into these following three levels.

Primary prevention

The step of prevention should be initially carried out before the fungal infestation and mycotoxin contamination. This level of prevention is the most important and effective plan for reducing fungal growth and mycotoxin production. Several practices have been recommended to keep the conditions unfavorable for any fungal growth. These include:

- development of fungal resistant varieties of growing plants;
- control field infection by fungi of planting crops;
- making schedule for suitable pre-harvest, harvest and post-harvest;
- lowering moisture content of plant seeds, after post harvesting and during storage;
- Store commodities at low temperature whenever possible;
- using fungicides and preservatives against fungal growth;
- control insect infestation in stored bulk grains with approved insecticides.

Secondary prevention

If the invasion of some fungi begins in commodities at early phase, at this level prevention will then be required. The existing toxigenic-fungi should be eliminated or its growth to be stopped to prevent further deterioration and mycotoxin contamination. Several measures are suggested as follows:

- Stop growth of infested fungi by re-drying the products;
- Removal of contaminated seeds;
- Inactivation or detoxification of mycotoxins contaminated;
- Protect stored products from any conditions which favour continuing fungal growth.

Tertiary prevention

Once the products are heavily infested by toxic fungi, the primary and secondary preventions would not be then feasible. Any action would not be as effective as the practices mentioned above, since it will be quite late to completely stop toxic fungi and reduce their toxin formation. However, some measures should be done to prevent the transfer of fungi and their health hazardous toxins highly contaminated in products into our daily foods and environment. For example, peanut oil extracted from poor-graded peanut seeds always contains very high levels of aflatoxins and the oil-soluble toxin has to be eliminated by absorption and alkalinization during oilrefining process. Only a few practices are recommended:

- Complete destruction of the contaminated products;
- Detoxification or destruction of mycotoxins to the minimal level.

Since aflatoxin is the most well-known mycotoxin ever throughly studied and its prevention and control has been most successfully practiced in various countries, therefore, this paper will focus on such practices in certain detail for the prevention and control of aflatoxins mycotoxin contamination. Successful development will bring a great impact for the increased production of crops and safe and nutritious foods around the world. A number of researchers have been working on *A.flavus*-resistant or tolerant varieties of corn and peanut. It has been clear that the fungal-resistance of each variety is genotypic. However, the resistance to invasion of *A. flavus* has been attributed to several biochemicals, environmental and physical factors. Uncontrollable factors could bring the failure in the utilization of selected fungal-resistant variety, as shown by laboratory screening, in the field.

Davis and his co-workers reported the survey and comparison of aflatoxin contamination in up to 215 corn hybrids grown in Alabama, USA during 1976-81. Unfortunately, they could not find any hybrid tested resistant to aflatoxin formation. They were convinced that significant aflatoxin levels generally accompanied stress caused by high temperature, low rainfall, low moisture-holding capacity of sandy soils and insect infestation.

A differential pathogenic capacity of various toxigenic strains of *A.flavus* have been observed . Some strains would require physical damage for their infestation and

others would not. The association of mycotoxin production and physical damage to grain and drought during grain ripening indicates *that Aspergillus spp*. are weak pathogens. During long grain storage, the biochemical activity of grain is much reduced, while invasion of storage fungi and mycotoxin contamination would increase. More data is needed on the biochemistry and pathogenesis of toxigenic fungi to understand and evaluate their genotype. The germination and viability of maize seeds could be affected by attack of *Aspergillus* and *Penicillium species* and their fungal infestation have been found to be different among maize genotypes.

Similarly, genotypes of peanut and biochemical properties of its seed such as tannin content, thin pericarp, small amount of cuticular wax and chemical composition of the pericarps and embryos have been shown to inhibit fungal invasion by *A. flavus* and aflatoxin formation.

Recently, antifungal enzymes, chitinase and B-1, 3-glucanase, found in a number of plant seeds, may act as defense against pathogenic fungi, since chitin and glucan are major polymeric components of many fungal cell walls. Such polysaccharides in fungal cell wall could be enzymically hydrolysed into smaller products resulting the damage or killing of fungal mycelia or spores. The role of these enzymes for genotype evaluation is now being studied. It is foreseen that seeds rich in such antifungal enzymes likely resist the infestation of fungi. If so, the seeds for breeding would be easily screened out and used a stock one.

Even there are many technical problems in searching for the "super" plant against pathogenicity, the development of fungal-resistant plant varieties utilizing genetic resistance to mycotoxin contamination is still possible and encouraged.

Fungal Growth Inhibition

How to prevent growth and invasion of pathogenic fungi in agricultural commodities is very important in preventing mycotoxin contamination. The inhibition of fungal growth can be achieved by physical, chemical and biological treatments

- Physical treatment. After the crops have been harvested, drying and proper storage and suitable transportation of the commodities are of prime importance. Several flavourable factors contribute to the growth of fungi and aflatoxin production, namely high moisture content, humid climate, warm

temperature (25-40°C), insect infestation and pes damage. Many means and measures to prevention of fungal contamination have been emphasized and practically done.

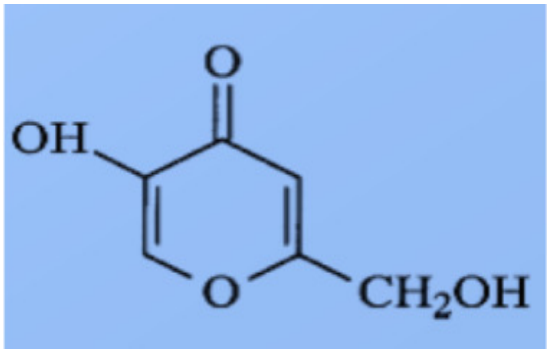
- Drying seeds and commodities to the safe moisture levels (<9% for peanut kernel, and < 13.5% for corn) .
- Maintenance of the container or warehouse at low temperature and humidity.
- Keep out insects and pests from the storage
- Gamma-irradiation of large-scale commodities.
- Chemical treatment with synthetic fungicides.
- Organic acids: acetic acid, propionic acid, butyric acid, malonic acid , benzoic acid, asorbic acid, lactic acid, citric acid and their sodium salts.
- Sodium chloride
- Benzoic acid derivatives: Onitrobenzoate, O-aminobenzoate, paminobenzoate, benzocain (ethly aminobenzoate), ethyl benzoate, methyl benzoate and aspirin (O-acetoxy benzoic acid)
- Potassium sulfite and potassium fluoride
- Fumigant: ammonia and phosphine .
- Treatment with natural products from plants or herbs.

1.4 Kojic Acid

Kojic acid (KA) is a secondary metabolite produced by some species of fungi from the genera *Aspergillus, Penicillium* and *Acetobacter,* it is produced biologically by different types of fungi during aerobic fermentation using various substrates. The name 'Kojic acid' (which was originally known as Koji acid) was derived from "Koji", the fungus starter orinoculum used in oriental food fermentations for many centuries. Kojic acid was originaly isolated in Japan by Saito in 1907 from mycelia of *Aspergillus oryzae* grown on steamed rice. This rice is called "koji" in Japanese, and this name was given to that organic compound by Yabuta in 1913. Its chemical structure was then extensively investigated and defined as 5-hydroxy-2-hydroxymethyl-g-pyrone (Yabuta, 1924). Chemical synonyms of kojic acid are known as 5-hydroxy-2- hydroxymethyl-4H-pyran-4-one (Nandan and Polasa, 1985) and 5-hydroxy-2-hydroxymethyl-4-pyrone (Kahn et al., 1995).

The properties of kojic acid

Kojic acid crystallises in the form of colourless, prismatic needles that sublime in vacuum without any changes. Mean while, the melting point of kojic acid ranges from 151°C - 154°C (Ohyama and Mishima, 1990). Kojic acid is soluble in water, ethanol and ethyl acetate. On the contrary, it is less soluble in ether, alcohol ether mixture, chloroform and pyridine (Wilson, 1971) . The molecular weight of kojic acid, as determined by the cryoscopic method for a formula of C6H6O4, is 142.1 (Uchino et al.,1988) . Kojic acid has a maximum peak of ultraviolet absorption spectra at 280 -284 nm (Choi et al., 2002; Watanabe-Akanuma et al., 2007) .The chemical structure of kojic acid is shown below indicates that:



Kojic acid

Molecular formula- $C_6H_6O_4$
CAS NO- 501-30-4
Melting Point- 151-154°C,
Molecular Weight- 142.1

Kojic acid is classified as a multifunctional, reactive g- pyrone with weakly acidic properties. It is reactive at every position on the ring and a number of products which have values in industrial chemistry, such as metal chelates, pyridones, pyridines, ethers, azodyes, mannich base, and the products of cyanoethylation can be formed from kojic acid (Ichimoto et al., 1965; Wilson, 1971). Numerous chemical reactions of kojic acid have been studied over the decades since its isolation. At carbon 5 positions, the hydroxyl group acts as a weak acid, which is capable to form

salts with few metals such as sodium, zinc, copper, calcium, nickel and cadmium (Crueger and Crueger, 1984). Kojic acid is produced by *Aspergillus* spp and *Penicillium* spp., belonging mainly to the *flavus-oryzaetamarii* groups. Among them, *A.flavus* (Basappa et al., 1970; Ariff et al., 1996), *A.oryzae* (Kwak and Rhee, 1992; Takamizawa et al., 1996), *A.tamarii* (Gould, 1938) and *A. parasiticus* (Nandan and Polasa, 1985; Coupland and Niehaus, 1987; El-Aasar, 2006) were reported to have the ability to produce large amounts of Kojic acid. Although, several potential Kojic acid producing strains have been isolated, very little attention has been paid to the improvement of the strains, either through mutation or genetic engineering techniques. Details of industrial techniques of Kojic acid fermentation are rarely revealed since they comprise the proprietary know-how of each producing company, *A.niger* can produce a variety of mycotoxins including oxalic acid, kojic acid, and cyclic pentapeptides called malformins (Kobbe et al., 1977; Wilson, 1966).

There are varieties of culture media used for production of Kojic acid such as:

- Modified Czapek –Dox medium
- The medium of Tadera
- Yeast extract sucrose medium

Carbon sources

A variety of carbon containing substrates may be used as carbon sources for Kojic acid fermentation .These substrates include starch, sucrose, maltose, glucose,fructose, mannose, galactose, xylose, arabinose, sorbitol, acetate, ethanol, glycerol and arabinose (Arnstein and Bentley, 1956; Wilson, 1971; Burdock et al., 2001; Rosfarizan and Ariffv 2007).

Nitrogen sources

There are variations in nitrogen sources chosen for Kojic acid fermentation from several strains of *Aspergillus spp*. Kitada et al. 1967 reported that organic nitrogen sources are generally better than inorganic nitrogen sources for Kojic acid fermentation. Complex organic nitrogen sources such as peptone and yeast extract may contain vitamins, which act as a precursor for Kojic acid production. Furthermore, some organic nitrogen sources have a good buffering system, while inorganic nitrogen sources, such as ammonia, excessively reduce the culture pH during NH4+ absorption. Low pH may influence the synthesis of Kojic acid during

fermentation and inhibit its growth. Yeast extract has been reported to be the most favourable organic nitrogen source for kojic acid production as compared to peptone and polypeptone (Wei et al.,1991; Ariff et al., 1996) . However, Kitada et al. (1967) and Coupland and Niehaus (1987) claimed that peptone is better than yeast extract for Kojic acid production. The presence of important growth factors, such as vitamins and oligoelements in specific nitrogen sources, also plays an important role in enhancing Kojic acid production (Gad, 2003). Kojic acid reversibly affects thyroid function primarily by inhibiting iodine uptake, leading to decreases in T_3 and T_4 and increase in TSH. Increased TSH from pituitary gland in turn stimulates thyroid hyperplasia. Several lines of evidence indicate that the proliferative effects of kojic acid on thyroid are not related to a genotoxic pathway. The risk of functional inhibition of iodine uptake and its metabolism (organification) and thyroid tumor induction by kojic acid in humans appears to be extremely low.

Applications

Whitening of skin, Antimicrobial, Antineoplastic, Analytical chemical, Chelating agents, Precursor for flavor enhancers, Conjugated with chitosan and amino acids for higher tyrosinase inhibition activity.

This organic compound (Kojic acid) continue toattract attention because of its economic potential in the field of , Medicine, Food, Cosmoteic and agricultural products.

Food industry

Kojic acid is used as an inhibitor of polyohenol oxidase, an enzyme responsible for the blackening of agricultural products (prevent oxidative browning).

Medicine and Cosmetic

Kojic acid is used mainly because it can eliminate free radicals and thus act as antioxidant. Kojic acid in the the form of gels and soaps is to achieve skin lightening effect by inhibiting the enzyme tyrosinase there by reducing the production of melanin formation, its also blocks the formation of pigmentation and even out skin tone, at present is introduced into various kinds of cosmoteics like curing freckles , lightining age spots on the skin , acne and stretch marks due to obesity and pregnancy.

1.5 Cytotoxic and cell proliferation assay

Cell viability and proliferation can be determined by three different methods as described below:

a. MTT Assay

Principle of assay:

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. isopropanol) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

b. Neutral red (NR) uptake assay

The cytotoxicity neutral red test is based on the abi0.lity of live cells to uptake and bind neutral red (NR).

NR is a positively charged dye that easily diffuses through the cellular membrane of the cells, accumulates in the cellular cytoplasma and is stored in the acidic environment of lysosomes. The principle of the test consists in the fact that NR are able to adsorb and bind only live cells while this ability declines in damaged or dead cells. The amount of accumulated NR is thus directly proportional to the amount of live cells in the cell cultur. The cytotoxicity neutral red test is based on the ability of live cells to uptake and bind neutral red (*NR*). NR is a positively charged dye that easily diffuses through the cellular membrane of the cells, accumulates in the cellular cytoplasma and is stored in the acidic environment of lysosomes. The principle of the test consists in the fact that NR are able to adsorb and bind only live cells while this ability declines in damaged or dead cells. The amount of accumulated NR is thus directly proportional to the amount of live cells in the cell culture.

The neutral red (NR) assay procedure is a cell survival/viability It is generally performed on adherent cells. NR is a weak cationic dye that readily penetrates the cell membrane and accumulates intracellular in lysosomes (lysosomal pH , cytoplasmic pH), where it binds with anionic sites to the lysosomal matrix (G. Griffon, et al. 1995). Changes of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such alterations brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells, which is the basis of the assay. The quantity of dye incorporated into cells is measured by spectrometry at 540 nm, and is directly proportional to the number of cells with an intact membrane. The assay can be used to evaluate cytotoxicity by determination of the IC50 (50% inhibiting concentration(. The NR assay is based on the ability of viable cells to incorporate and bind the supravital dye NR in lysosomes, via active transport. NR is a weak cationic dye and penetrates cell membranes by nonionic diffusion, subsequently binding intracellular to sites of the lysosome. Cell membrane injuries decrease the uptake and retention of NR.

c. Propidium iodide (PI) assay

Propidium iodide solution (PI) is a membrane impermeant dye and an intercalating agent.

When added to a cell population, PI will penetrate cells with ruptured membranes to bind to DNA providing a strong fluorescent signal which can be readily visualised using fluorescence imaging or micropscopy. As such, it is a commonly used as a dependable marker for ‘dead cells’ in a population of ‘live cells’. Fluorescence-only approaches to PI imaging are normally well suited for counting the ‘dead cells’ but then require a work around to measure the live cell population. This can mean either a separate fluorescent dye and imaging channel or commonly, taking a separate sample, rupturing the membranes and then doing a separate PI count on this second sample. By being forced to double up the sample clearly doubles theworkload and associated expence but crucially looses a direct link between what is ‘dead’ and what is ‘live’ on a single image The Cellometer Vision (figure 1) uses a novel brightfield plus fluorescent approach to PI imaging to display clearly, on a single sample, the cells which are stained with PI and which are not. Cell counting is automated and rapid,

taking between 30 – 60 seconds for a count and results are clearly displayed on screen.

2. LITERATURE REVIEW

Blumenthal CZ 2004 studied on *Aspergillus niger, Aspergillus oryzae* , and *Trichoderma reesei* are three important production organisms used in industrial fermentations. Several of the fungal secondary metabolites produced by selected strains of these three fungi are capable of eliciting toxicity in animals. Among those toxic substances are the well-known mycotoxins 3-nitropropionic acid and ochratoxin A. However, many others, such as Kojic acid, may not be true mycotoxins. The production, extraction, chemical structure, and the toxicity (expressed as LD(50)) of these substances were reviewed. Production of toxic secondary metabolites in *A.niger, A.oryzae,* and T. reesei is strain-specific and environment-dependent. Considering all of the safety measures taken in the industrial production process, these three fungal species are safe to use. The recently revised JECFA specification for mycotoxins in food enzyme preparations was also discussed. The extent of mycotoxin tests in food enzyme preparations should be judged on a case-by-case basis, through a careful evaluation based on knowledge of taxonomy, biochemistry, and genetics. In many cases, the testing scope at the level of genus should be sufficient. In other cases, the scope can even be further narrowed based on scientific knowledge and assessment.

D'Mello and Macdonald 1998 investigated that mycotoxins are those secondary metabolites of fungi that have the capacity to impair animal health and productivity (D'Mello and Macdonald, 1998). The diverse effects precipitated by these compounds are conventionally considered under the generic term "mycotoxicosis", and include distinct syndromes as well as non-specific conditions, also indicates the fungal species associated with the production of these contaminants. Mycotoxin contamination of forages and cereals frequently occurs in the field following infection of plants with particular pathogenic fungi or with symbiotic endophytes. Contamination may also occur during processing and storage of harvested products and feed whenever environmental conditions are appropriate for spoilage fungi. Moisture content and ambient temperature are key determinants of

fungal colonization and mycotoxin production. It is conventional to subdivide toxigenic fungi into "field" (or plant-pathogenic) and "storage" (or saprophytic/spoilage) organisms. Claviceps, Neotyphodium, Fusarium and Alternaria are classical representatives of field fungi while *Aspergillus* and *Penicillium* exemplify storage organisms. Mycotoxigenic species may be further distinguished on the basis of geographical prevalence, reflecting specific environmental requirements for growth and secondary metabolism. Thus, *Aspergillus flavus, A. parasiticus* and A. ochraceus readily proliferate under warm, humid conditions, while *Penicillium expansum* and *P. verrucosum* are essentially temperate fungi. Consequently, the *Aspergillus* mycotoxins predominate in plant products emanating from the tropics and other warm regions, while the *Penicillium* mycotoxins occur widely in temperate foods, particularly cereal grains. *Fusarium fungi* are more ubiquitous, but even this genus contains toxigenic species that are almost exclusively associated with cereals from warm countries.

Patricia A and Murphy et al 2006 reported that unavoidable, natural contaminants in foods may have either chemical or biological origin. Mycotoxins toxic secondary metabolites of fungi are biological in origin. Despite efforts to control fungal contamination, toxigenic fungi are ubiquitous in nature and occur regularly in worldwide food supplies due to mold infestation of susceptible agricultural products, such as cereal grains, nuts, and fruits. Thousands of mycotoxins exist, but only a few present significant food safety challenges. The natural fungal flora associated with foods is dominated by three genera *Aspergillus, Fusarium*, and *Penicillium*, which except for the *Fusarium* plant pathogens, may include commensals as well as pathogens. The chemical structures of mycotoxins produced by these fungi are very diverse,as are the characteristics of the mycotoxicoses. Ergotism is the oldest identified mycotoxicosis in humans. This mycotoxin represents a group of alkaloids that grow on the heads of grasses, such as wheat and rye. Ergot was responsible for a disease of the Middle Ages known as “St. Anthony’s Fire,” so named for the burning sensation cause din victims’ limbs.The Spartans apparently suffered an ergot epidemic in 430 B.C.. Ergotism has also been associated with the Salemwitch trials in the 1600s in Massachusetts. More recent outbreaks, associated with economic up heaval and war, have occurred in Russia (1924 and 1944), Ireland (1929) ,France (1953), and Ehiopia (1978). Although ergot poisoning continues to pose achallenge for the

live stock industry, the toxin is less of achallenge for the food industry because current food quality control procedures screen out ergot-infected grains. Of the thousands of existing mycotoxins, a few hundred are associated with food and only a handful present food safety challenges to the farm-to-fork food continuum. At the farmlevel, mold growth can result in reduced crop yields and livestock productivity stemming from illness or death due to consumption of contaminated feed. In food manufacturing, destruction of mycotoxins by conventional food processing is difficult because they are typically highly resistant and detection is complicated due to limitations in analytical methodology. In the marketplace, mycotoxins can be a hurdle to international trade, leading to increase d regulation of foods and feeds that may contain them and removal from the market of commodities not meeting regulatory limits. when present in foods in sufficiently high levels, these fungal metabolites can have toxic effects that range from acute (for example, liver or kidney deterioration), to chronic (for example, liver cancer), mutagenic, and teratogenic; and resulting symptoms range from skin irritation to immune suppression, birth defects, neurotoxicity,and death(ICMSF 1996). Aflatoxin B1 (AFB1), fumonisins, and patulin are suspected human carcinogens.Deoxynivalenol and other trichothecenes aswell as AFB1 are likely to exert immunosuppressive effects, and fumonisin B1(FB1) may contribute to neural tube defects. Renal dysfunction due to ochratoxin A exposure (suspected in Balkan endemic nephropathy) is also apotentially significant problem,especially as this could exacerbate impaired renal function in individuals with diabetes, a burgeoning worldwide epidemic that is highly likely to grow. There is also uncertainty related to the effects of chronic low-level, long-term exposure tosingle and/or multiple mycotoxins, which may be the case even for those individuals consuming adiverse diet. Environmental factors affect mycotoxin presence in raw and stored commodities. Data on optimal temperature and water activity for toxin production by *Aspergillus, Penicillium,* and *Fusarium spp*. Traditionally, control of mycotoxin contamination of foods has been attempted through control of water activity, pH and quality control of incoming ingredients. Novel control avenues are emerging, including availability of genetically modified grains with increased insect resistance and, thus, lowered rates of fungal infection; improved management of grain ingredients; and inclusion of controls for mycotoxins in food manufacturing Hazard Analysisand Critical Control Point(ACCP) plans. Genotyping techniques have shed new light on mycotoxin producing fungi and

provided the foundation for advances in detection methodology. Historically, fungi have been identified on the basis of traditional taxonomic characteristics (for morphological features); more recently, the tools of molecular biology have enabled genetic analysis and classification on on the basis of nucleic acid sequence.Since analytical methods for detecting mycotoxin shave become more prevalent, sensitive,andspecific ,surveillance of foods for mycotoxin contamination has become more common.

Wojciech Dabrosand Anna Kordowiak 2007 proved the biochemical activity and morphology of control and streptozotocin-diabetic rat liver Golgi complexes were previously investigated by us under influenc of some vanadium [V(IV)] compounds. The effectiveness of these derivatives depends on the kind of complexing ligands. This paper presents the investigation of the effect of bipyridyl, the ligand of a new vanadium compound, tested by us with maltol and Kojic acid. The three ligands alone action was tested under the same experimental conditions as in the case of whole compounds with vanadium and applied to liver Golgi complexes of control rats. A preliminary study for maltol and Kojic acid had been previously carried out by us parallel with tests of whole vanadium complexes, but valuable differences in biological action found in our condition of experiments suggested the extension of studies to include the two above-mentioned ligands and to compare the effects of the three investigated ligands. The supplementary part of the experiment focused mainly on the ultrastructure of Golgi complexes in hepatocytes. Four groups of animals were used: C-control rats,C + M (maltol), C + (ka)2 (kojic acid) and C + (bpy)2 (bipyridyl). The control rats received 0.09M NaCl as drinking liquid; all the other animals were given 3.6 mmol/L of appropriate ligand solution in 0.09M NaCl during 7 days. All the animals survived the experiments. Only in group C+(bpy)2 did the authors observe statistically significant differences as compared with the controls (group C). The differences were detected in physiological studies and manifested as body weight decreased by approximately 20% during the experiment, lower liquid.

Russell R and M Paterson 2010 assessesed the impact of climate change on mycotoxins in food. Climate change is an accepted probability by most scientists. Favourable temperature and water activity are crucial for mycotoxigenic fungi and mycotoxin production. Fungal diseases of crops provide relevant information for pre-

harvest mycotoxin contamination. However, the mycotoxin issue also involves post-harvest scenarios. There are no data on how mycotoxins affect competing organisms in crop ecosystems. In general, if the temperature increases in cool or temperate climates, the relevant countries may become more liable to aflatoxins. Tropical countries may become too inhospitable for conventional fungal growth and mycotoxin production. Currently cold regions may become liable to temperate problems concerning ochratoxin. A, patulin and Fusarium toxins (e.g. deoxynivalenol). Regions which can afford to control the environment of storage facilities may be able to avoid post-harvest problems but at high additional cost. There appears to be a lack of awareness of the issue in some non-European countries. The era will provide numerous challenges for mycotoxicologists.

Hee Youn Chee and Eun Hee 2003 proved that Kojic acid has antifungal activity against the human pathogenic fungi including *Candida albicans*, *Cryptococcus neoformans* and *Trichophyton rubrum*. For *C. albicans, C. neoformans* and *T. rubrum,* the MIC (minimum inhibitory concentration) of Kojic acid was 640, 80 and 160 mg/ml, respectively. In *C.neoformans*, melanin-producing yeast, Kojic acid treated nonmelanized cell was more susceptible to magainin than melanized cell, suggesting melanin give a protective function against microbial peptide.

El-Aasar SA 2006 screened five local *Aspergillus* species for Kojic acid production statically on four proposed kojic acid producing synthetic medium. *A. parasiticus* was found to be the highly active organism for acid production. The study implicated the optimization of different culture conditions of fermentation medium; initial pH, incubation temperature, carbon source concentration (glucose, sucrose & sugar beet molasses), nitrogen source concentration (yeast extract, peptone, ammonium sulphate, ammonium nitrate & their combinations) and changes of Kojic acid yield in static and rotary shaking culture. The highest level of kojic acid (34.38 g L-1) was obtained by *A. parasiticus* using fermentation medium of 6% glucose, 1% yeast extract with initial pH 5 and incubated at 28°C for 10 days under rotary shaking culture (220 rpm). Antimicrobial activity of Kojic acid was compared to some antibiotics against three Gram – ve, three Gram + ve bacteria and two strains of Candida. They cocluded that enhanced production of Kojic acid was successfully

achieved by optimizing the fermentation conditions for growth and productivity of *A. parasiticus.*

Ana Paula D Rodrigues et al 2011 demonstrated skin whitening, antioxidant, and antitumour properties of Kojic acid (KA) a secondary metabolite isolated from *Aspergillus fung*. The aim of their study was to analyse whether Kojic acid (KA) affects functional properties related to macrophage activation, such as phagocytosis and spreading ability over a substrate. Treatment of resident macrophages with 50 mg/ml KA for 1 h induced both morphological and physiological alterations in cells. Immunofluorescence microscopy revealed enhanced cell spreading and an increase in cell surface exposure, associated with a rearrangement of microtubules, actin filaments and intermediate filaments. KA also potentiated phagocytosis by macrophages, as demonstrated by the increase in phagocytic activity towards yeast, when compared to untreated cells. Kojic acid increased the production of ROS (reactive oxygen species), but not NO (nitric oxide) production. Three tests were used to assess cell viability; MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], NR (neutral red) uptake and PI (propidium iodide) exclusion test, which showed that macrophages maintain their viability following Kojic acid treatment. Results indicate that Kojic acid can modulate macrophage activation through cytoskeleton rearrangement, increase cell surface exposure, enhance the phagocytic process and ROS production. The study demonstrates a new role for Kojic acid as a macrophage activator.

Rashad M Saleh et al 2011 focused on the production of antibacterial compound from *Trichoderma spp.* Screening of antibacterial activities in some *Trichoderma* spp. was investigated using CYS80 medium. *Trichoderma reesei* and *Trichoderma viride* were highly effective toward human-pathogenic bacteria tested. *T. viride* and *T. reesei* were separately applied on Sephacryl S–200 column. Column fractions No. 56 to 64 for *T.viride* and fractions No. 57 to 66 for *T.reesei* had inhibitory effect against the most pathogenic bacteria examined. T. reesei and T. viride Sephacryl S-200 fractions with antibacterial activity were analyzed by Gas chromatography–mass spectrometry (GC- MS). The product with highest peak (95%), using different libraries, was Kojic acid. The yield of Kojic acid crystals from *T.reesei* and *T.viride* Sephacryl S-200 fractions were 3 and 5 g/L, respectively. Physical

analysis of Kojic acid with respect to UV, IR, 1HNMR analysis and melting point was examined. The minimum inhibitory concentration (MIC) of Kojic acid and augmentin, as control, against human-pathogenic bacteria were evaluated. Kojic acid and augmentin showed a similar time-killing kinetics with human-pathogenic bacteria. The level of Kojic acid increased with decreased level of reducing sugar during the growth of *T. reesei* and *T. viride* suggesting that the enzyme system for the synthesis of kojic acid found in the cell of these fungi.

George A Burdock et al 2000 reviwed and reported that Kojic acid is a fungal metabolite commonly produced by many species of *Aspergillus, Acetobacter,* and *Penicillium*. The *Aspergillus flavus* group has tradiationally been used in the production of a number of foods, including miso (soybean paste), shoyu (soy sauce), and sake. Kojic acid is widely used as a food additive for preventing enzymatic browning, and in cosmetic preparations as a skin-lightening or bleaching agent. Because Kojic acid is often produced during the fermentation of historically used dietary staples, it has a long history of consumption. Various types of compounds, such as glucose, sucrose, acetate, ethanol, arabinose, and xylose, have been used as carbon sources for kojic acid production. Different *Aspergillus* species are known to produce variable amounts of kojic acid. The mechanism of action of Kojic acid is well defined and it has been shown to act as a competitive and reversible inhibitor of animal and plant polyphenol oxidases, xanthine oxidase, and L- and some L-amino acid oxidases. The structure of kojic acid indicates a relatively simple route of metabolism much like dietary hexoses. Acute or subchronic toxicity resulting from an oral dose has not been reported, but convulsions may occur if Kojic acid is injected. Results of mutagenicity studies are mixed, but in the in vivo mammalian dominant lethal assay, Kojic acid was proven negative. Continuous administration of high doses of Kojic acid in mice resulted in induction of thyroid adenomas in both sexes. Kojic acid reversibly affects thyroid function primarily by inhibiting iodine uptake, leading to decreases in T_3 and T_4 and increase in TSH. Increased TSH from pituitary gland in turn stimulates thyroid hyperplasia. Several lines of evidence indicate that the proliferative effects of Kojic acid on thyroid are not related to a genotoxic pathway. The risk of functional inhibition of iodine uptake and its metabolism (organification) and thyroid tumor induction by Kojic acid in humans appears to be extremely low. Based on the literature reviewed and discussed here, common contaminant

consumption of Kojic acid at levels normally found in food does not present a concern for safety.

Jong H. Kim et al 2011, proved that the natural compounds that pose no significant medical or environmental side effects are potential sources of antifungal agents, either in their nascent form or as structural backbones for more effective derivatives. Kojic acid (KA) is one such compound. It is a natural by-product of fungal fermentation commonly employed by food and cosmetic industries. We show that Kojic acid greatly lowers minimum inhibitory (MIC) or fungicidal (MFC) concentrations of commercial medicinal and agricultural antifungal agents, amphotericin B (AMB) and strobilurin, respectively, against pathogenic yeasts and filamentous fungi. Assays using two mitogen-activated protein kinase (MAPK) mutants, i.e., sakAΔ, mpkCΔ, of *Aspergillus fumigatus*, an agent for human invasive aspergillosis, with hydrogen peroxide (H2O2) or AMB indicate such chemo sensitizing activity of Kojic acid is most conceivably through disruption of fungal antioxidation systems. Kojic acid could be developed as a chemosensitizer to enhance efficacy of certain conventional antifungaldrugs or fungicides.

Rosfarizan Mohamad et al 2010 proved Kojic acid, 5-hydroxy-2-hydroxymethyl-g-pyrone, has many potential industrial applications. In this review, the properties and diverse applications of kojic acid in industries are described. The review also discusses the advance in Kojic acid fermentation, focusing on the process development in microorganisms and strain selection, medium and culture optimization, as well as fermentation modes for commercially viable industrial scale production.

Yu-Hua Chen et al 2013 Synthesized and examined three classes of kojic acid derivatives for their antiproliferative activity against HeLa cells. Both **8b** and **11** co-treated with copper ion exhibited synergistic effect on the HeLa cell growth inhibition with GI_{50} values of 11.9 and 7.1 μM, respectively. Flow cytometric analysis of HeLa cells revealed that **11**-Cu co-treatment induced the sub-G1 arrest in a dose-dependent manner, suggesting that the growth-inhibitory effect is attributed to DNA fragmentation. Moreover, western blot of HeLa cells cytosolic extracts displayed the cleavage of the 116-kDa protein poly(ADP-ribose) polymerase and activation of caspase-3 by the reduced level of the 32-kDa proenzyme, indicating that the caspase-

dependent apoptotic pathway was involved. Further they demonstrated that MAPK pathway regulators such as ERK and p38 were activated in response to**11**-Cu co-treatment, suggesting that the intracellular oxidative stress was dramatically stimulated by the copper ion.

Rosfarizan et al 1998 made a direct conversion of gelatinized sago starch into Kojic acid by *Aspergillus flavus* strain having amylolytic enzymes was carried out at two different scales of submerged batch fermentation in a 250-mL shake flask and in a 50-L stirred-tank fermentor. For comparison, fermentations were also carried out using glucose and glucose hydrolyzate from enzymic hydrolysis of sago starch as carbon sources. During Kojic acid fermentation of starch, starch was first hydrolyzed to glucose by the action of α-amylase and glucoamylase during active growth phase. The glucose remaining during the production phase (non-growing phase) was then converted to Kojic acid. Kojic acid production (23.5g/L) using 100 g/L sago starch in a shake flask was comparable to fermentation of glucose (31.5 g/L) and glucose hydrolyzate (27.9 g/L) but in the 50-L fermentor was greatly reduced due to non-optimal aeration conditions. Kojic acid production using glucose was higher in the 50-L fermentor than in the shake flask.

Satomi Kawagushi et al 2007 stated that Kojic acid is used as a cosmetic agent for skin whitening , information for its genotoxicity in *in vitro* assay is much complicated .in order to evaluate its genotoxic potentials *in vitro* they conducted comet assay in regular and acellular versions, chromosome aberration assay and TK mutation assay in human lymphoblastoid cells. Positive assay were obtained in all comet, chromosome aberration and TK mutation assays at almost identical concenteration in both TK6 and WTK1 cells. In the acellular comet assay , Kojic acid led to positive response at pH 12 and pH 13suggested that it induce DNA single strand breaks (SSBs). In the TK mutation assay Kojic acid increased the fraction of normal growing but not slowly growing mutants suggesting that observed gene mutation are due to point mutation with in TK locus but not growth structural changes that can form chromosome aberrations observed by a microscopy.

3. SCOPE AND AIM

Kojic acid is utilized in cosmaceutical industry for the purpose of skin whitening agent.

Kojic acid is reported to have anticancer and antimicrobial property. Few reports, demonstrate Kojic acid as toxic material. Many experiments are carried out to understand the toxicity.

In veiw of this current study was aimed to optimize the carbon and nitrogen sources need for the production of Kojic acid from a fungal species and to determine the antimicrobial potency and cytotoxicity of Kojic acid in selected skin cancer cell lines.

4. PLAN OF WORK

Collection of fungal strain (*Aspergillus niger* MTTC2208)

↓

Nutritional [carbon/nitrogen] optimization in media

↓

Fermentation of Kojic acid [submerged]

↓

Isolation of biosynthesized Kojic acid

↓

Confirmation and Quantification of biosynthesized Kojic acid

↓

Antimicrobial & Cytotoxic evaluation

5. MATERIALS AND METHODS

Table 2 : Materials

S. No	Chemicals/Culture/Instruments	Company
1.	Potato Dextrose Agar	Hi-Media
2.	Muller Hinton Agar	Hi-Media
3.	Glucose	Nice chemicals
4.	Sucrose	Nice chemicals
5.	Peptone	Hi-Media
6.	Ammonium Sulphate	Qualigens
7.	Sodium nitrate	Nice chemicals
8.	Potassium di hydrogen phosphate	Finar chemicals
9.	Magnesium Sulphate	Fischer
10.	Potassium chloride	Qualigens
11.	Ferrus sulphate	Nice chemicals
12.	Ethyl acetate	Nice chemicals
13.	Ferric chloride	Nice chemicals
14.	Standard antibiotic discs	Hi-Media
15.	Ethanol	Otto
16.	*Aspergillus niger MTCC 2208*	IMTECH, Chandigar
17.	*C.Albicans*	IMTECH, Chandigar
18.	*Escherichia coli*	Bose lab, Madurai
19.	*Pseudomonas aeruginosa*	Bose lab, Madurai
20.	*Bacillus subtilus*	Bose lab, Madurai
21.	*Staphylococcus aureus*	Bose lab, Madurai
22.	Rotary vacuum evaporator	Roteva
23.	Incubator	ILTC
24.	Autoclave	Equitron
25.	pH Meter	Systronics

6. METHODOLOGY

6.1 CULTURE

The selected fungal strain for the current study is *Aspergillus niger* MTCC 2208 and was procured from IMTECH, Chandigarh and maintained in CYA slants as per IMTECH guidelines.

6.2 CULTURE MAINTENANCE

Table 3: Composition of Czapek concentrate

S. No.	INGREDIENTS	QUANTITY
1.	Sodium nitrate	30.0 g
2.	Potassium chloride	5.0 g
3.	Magnesium sulphate	5.0 g
4.	Ferrus silphate	0.1 g
5.	Distilled water	100.0ml
6.	Ph	7.2

Table 4: Composition of Czapek yeast agar medium

S. No.	INGREDIENTS	QUANTITY
1.	Czepak concentrate	10.0 g
2.	Di potassium hydrogenphosphate	1.0 g
3.	Powdered yeast extract	5.0 g
4.	Sucrose	30.0 g
5.	Agar	15.0 g
6.	Distelled water	1.0 L

Czapek yeast agar medium was prepared and sterilized by autoclaving at 121 ° C for 15 minutes. The organism was streaked on the surface on the surface of CYA as per IMTECH,Chandigarh standard procedure, the plates were incubated at 25± 2 ° C for 24 hour. Further subculture was done in potato dextrose broth medium (pH 5.1). The seed culture for our expremental studies was done in PDB medium and the culture grown in was streaked in experimental media.

6.3 NUTRITIONAL [CARBON/NITROGEN] OPTIMIZATION IN MEDIA

Since carbon and nitrogen has significant role in biosynthesis of extracellular Kojic acid,sucrose, glucose, peptone [N_2 (O)] and ammonium sulphate [N_2 (IO)] were added in varied concentration to the Czapek- Dox medium [CD] which is a basic media recommended for Kojic acid biosynthesis in most of the protocols described earlier.

Table 5: Concentration of carbon and nitrogen utilized

S.No	Carbon source	Concentration g/l	Nitrogen sources	Concentration g/l
1.	Glucose	35 , 45 , 55	Peptone	5, 10 , 15
2.	Sucrose	35, 45 , 55	Ammonium sulphate	5, 10 , 15

6.4 FERMENTATION OF KOJIC ACID (SUBMERGED)

Fermentation for biosynthesising Kojic acid was done using submerged fermentation. Various runs were employed as described in table 6. Periodicaly subcultured 5ml of *A.niger* MTCC 2208 (inoculum) was added in sterilized modified czapek dox media previously loaded with carbon and nitrogen sources.The entire flask were incubated for 14 days at 25 ± 2° C.

Table 6: Scheme For Fermentation

S. No.	DESCRIPTION	RUNS
1.	Czapek- Dox (CD)	1
2.	Glucose + CD	3
3.	Sucrose + CD	3
4.	N_2 (O) + CD	3
5.	N_2 (IO) + CD	3
6.	Glucose +N_2(O) + CD	9
7.	Glucose+N_2 (IO)+CD	9
8.	Sucrose+N_2 (O)+CD	9
9.	Sucrose+N_2 (IO)+CD	9
10.	**Total**	49

6.5 ISOLATION OF KOJIC ACID

The mycelia obtained by the above runs were seprated by filteration through whattman filter paper no.1 filter paper. Equal volume of ethyl acetate was added to all flasks containing fermented broth. Then, the mixture was shaken in a separating funnel over night. The organic layers were seprated and collectd. The solvent was removed in vaccum using a rotarory vaccum evaporator. The residue was collected and weighed. The weight of resides were tabulated in table 7 and 8. Kojic acid obtained from all the 49 runs were pooled together and subjected to antimicrobial and cytotoxic evaluation.

6.6 QUANTIFICATION OF KOJIC ACID

Kojic acid produced in fermentation was estimated by reacting the properly diluted samples with 1% ferric chloride in 0.1 N Hcl. The absorbance at 505 nm was read and interpreted with Kojic acid standard curve the volumes were represented in table 7 and 8.

6.6.1 Standard curve

Kojic acid 10mg was dissolved in 10ml CD, 1ml of ferric chloride and 0.1N hydrochloric acid were added to the above solution, 100µ, 200µ, 400µ, 800µ, 1600µ, 3200µ, were taken and the absorbance was read at 505 nm. Graph was ploted concentration with absorbance shown in table 9 and graph 4.

6.7 ANTIMICROBIAL ACTIVITY – DISC DIFFUSION METHOD

Of the many media available, MHA and PDA were considered to be best for routine susceptibility testing of bacteria and antifungal for the following reasons :

- It shows acceptable batch to batch reproducibility for susceptibility testing.
- It gives satisfactory growth of most pathogens.

MHA and PDAwere prepared from available dehydrated base according to manufacturers instructions. Immediatelly after autoclaving , it allowed to cool in a 45-50 ºC water bath. The freshely prepared and cooled medium was poured inti glass petridishes on alevel, horizontal surface to give auniform depth of approximetaly 4mm. The agar medium was allowed to cool to room temperature. Representative sample of each batch of plates was examined for sterility by incubating at 30 to 35 º C for 24 hours or longer.

Whatman filter paper no.1 is used to prepare approximately 8mm in diameter, which was placed in petridish and sterilized in ahot air oven. These sterilized discs were then loaded with the component (Kojic acid) dissolved in methanol with the required concentration using micropipette and it was dried.

Penicilln G (10µg) was used as standard disc for bacteria.
Fluconazole (10µg) was used as standard disc for fungi
Methanol was used as a control.

The prepared disc were stored in air closed tight container in refrigerator until use. Inoculation of test plates. A sterile cotton swap was dipped into the culture *(Escherichia.coli, Staphylococcus aureus, Bacillus subtilus,P vulgaris, pseudomonas aeurgenosa, A.niger, C.albicans..).*

The swab were rotated several times and pressed firmly on the inside wall of the tube this will remove excess inoculums from the swab. The dried surface of Muller-Hinton agar plate was inoculated by streaking the swab over the sterile entire agar surface. This procedure is repeated by streaking two more times, rotating the plate 60 ^{0} each time to ensure an even distribution of inoculums. The lid may left after for 3-5 min, but no more than 15 min, to allow for any excess moisture to be absorbed before applying the drug impregenated discs. The disc is dispenced into inoculated agar plate using sterile forceps . Each disc was pressed down to ensure complete contact with the agar surface. The disc was distributed evenly so that they will not closer to each other. The plates were inverted and placed in an incubator set to 35 ^{o}C with in 15 minutes after the discs were applied. After 18 hours of incubation the plate was examined . The resulting zones of inhibition will be uniformally circular and there was a confluent lawn of growth. The diameter of the zones of complete inhibitions were measured. The zone were measured using a measure scale and tabulated in table 10.

6.8 CYTOTOXIC ACTIVITY

In vitro cytotoxicity of Kojic acid

Chemicals

3-(4,5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd., Mumbai, India.

Cell lines and Culture medium

3T3L1 cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 g/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 at 37µC until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture

flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions

For cytotoxicity studies, weighed test drug was separately dissolved in DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by MTT Assay

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used (Francis and Rita, 1986).

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 °l of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37° C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37° C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol

was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100\left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}}\right) \quad X\ 100$$

6.9 Spectral analysis

FT-IR spectrum of Kojic acid

Spectral analysis was carried out to determine the Kojic acid extracted from *Aspergillus niger* MTCC 2208 broth. The IR spectrum of standard Kojic acid and sample obtained after fermentation were compared and shown in figure 2,3 and interpreting the frequency on table 12.

7. RESULT AND DISCUSSION

7.1 PRELIMINARY RESULTS

Aspergillus niger MTCC 2208 organism was procured from IMTECH Chandigarh. The organism was subcultured in modified Czapek Dox medium. The modified Czepak broth medium was prepared using different carbon and nitrogen sources with different concentration explained in table 5. The organism was inoculated and incubated at 25 ± 2^{o} C for 14 days.

7.1.1 Crude extraction

Isolation of Kojic acid was done by filtering the biomass using Whatman filter paper no 1 and the supernatant was extracted with equal amount of organic solvent ethyl acetate, the solution was kept in shaker for overnight, two layer were formed, the organic layer was separated by separating funnel and evaporated by rotary vacuum evaporator .The biomass was dried and weighed by dry weight method.

7.1.2 Nutritional [Carbon/Nitrogen] Optimization in Media

The choice of carbon and nitrogen sources for the fermentation medium can be an important factor influencing the subsequent fermentation (Futamura et al 2001). In most fermentation processes, the concentration of the nitrogen and/or carbon source in the medium is increased to secure high productivity, there for, when selecting an optimum production medium, it is very important to select a medium yielding low cell concentration (Ariff et al 1996). From the above study the highest yield of kojic acid was obtained in the fermentation using glucose as a carbon source and peptone as an organic a nitrogen source when compared with sucrose and peptone. The highest production of kojic acid was obtained at concentration of 55g glucose and 5g of peptone as a nitrogen source and the yield was 1144.7µg, so the production of kojic acid increased with the increase of carbon source and decrease of nitrogen source, the weight of the biomass dose not have any effect on the quantity of kojic acid produced. The concentrations of kojic acid produced by different concentrations of carbon and nitrogen sources were shown in table 8.

7.1.2.1 Effect of carbon source in biosynthesizing Kojic acid

In this study glucose and sucrose are the two carbon sources utilized for the biosynthesis of kojic acid at varied concentrations of 35, 45, and 55 g/l. the highest yield of kojic acid was obtained in the fermentation using glucose as the carbon source followed by sucrose and fructose (Rosfarizan Mohamed et al 2010). Also some other study reported that the glucose concentration ranging from 25-150 g/l influenced the production of kojic acid synthesis (Kitada et al 1967). In this study czepak dox when combined with glucose concentrations 35,45 and 55 g/l achieved high biomass 3.6 g/l while no kojic acid was synthesized. As the same thing when sucrose as carbon source added with czepak dox medium for fermentation maximum biomass and no kojic acid synthesis was achieved. This shows carbon source alone is not worthwhile for the synthesis of kojic acid production. When the carbon sources combined with nitrogen biomass as well as kojic acid synthesis was seen.

When carbon sources at 45 and 55 g/l combined with 5-15 g/l concentrations of nitrogen organic and inorganic, reported to produce high yield of biomass and kojic acid. In the above combinations if nitrogen source is high the effect of kojic acid production becomes less but biomass concentration obtained was high. This shows the

production relies upon the concentration of carbon as well as nitrogen sources. Some other study explains that sucrose was the best source for producing kojic acid (Wei et al 1991), but from this study it is under stood that concentration plays a major role. Nitrogen organic with glucose profounds yield high quantity of kojic acid compared to nitrogen inorganic which is negligible.

7.1.2.2 Effect of nitrogen source in biosynthesizing Kojic acid

Organic nitrogen source always contains important growth factors which plays an important role in enhancing biomass production (Gad 2003). So It becomes mandatory for nitrogen to be considered as a major source in growth but limited for kojic acid synthesis as per the result obtained in this study. Hence nitrogen limited fermentation is required for biosynthesizing kojic acid (Ariff et al 1996). In this study peptone and ammonium sulphate were utilized as nitrogen organic and nitrogen inorganic sources respectively in varied concentrations of 5, 10, and 15 g/l. nitrogen alone in combination with czepak dox medium doesn't produced any kojic acid and biomass as well. When least concentration of nitrogen organic/inorganic was added with glucose/sucrose, it yield high quantity of kojic acid but negligible at high concentrations. This confirms that low concentrations are needed to perform the biosynthesis of kojic acid.

WEIGHT OF BIOMASS AND CRUDE EXTRACT

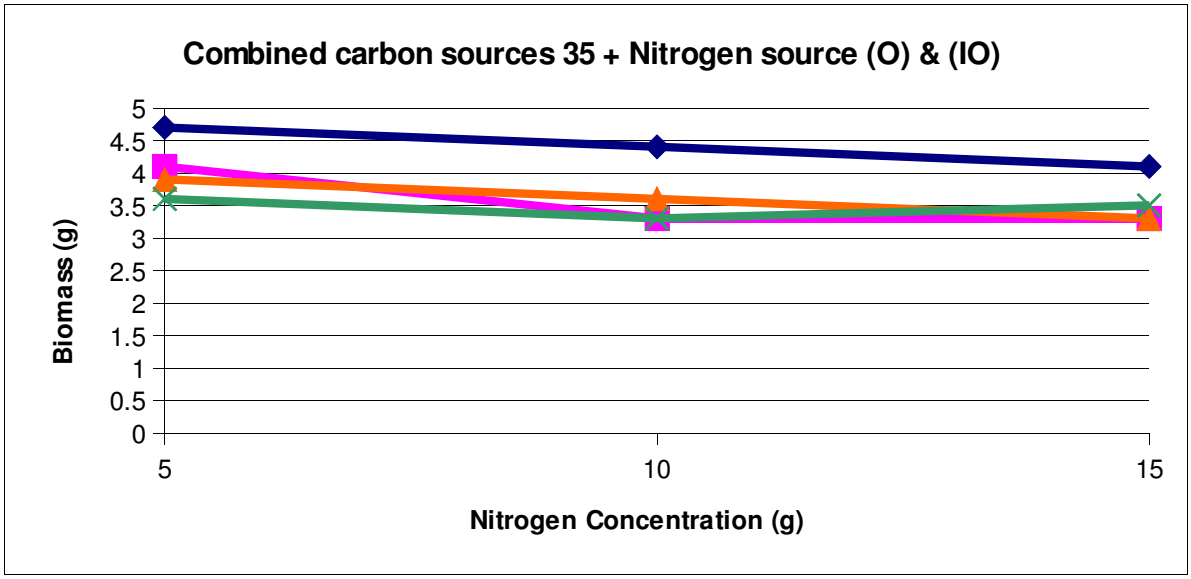
Table 7: Concentration (g) of carbon and nitrogen sources added to Czepak dox media

S. No.	Description of runs		Biomass(g/l)	Quantity of Kojic acid(µg/l)
1.	Czepak dox (1L)		1.3	-
2.	Czepak dox +Glucose	35	3.2	-
		45	3.6	-
		55	3.3	-
3.	Czepak dox+Sucrose	35	3.1	-
		45	3.3	-
		55	3.3	-
4.	Czepak dox+Nirogen (O)	5	1.4	-
		10	1.3	-
		15	1.1	-
5.		5	1.3	-

	Czepak dox+Nitrogen (IO)	10	1.1	-
		15	1.1	-

Table 8 : Concentration (g) of combined carbon and nitrogen sources added to Czepak dox media

S. No	Description of runs			Biomass (g/l)	Quantity of Kojicacid(µg/l)
1.	Czepak dox +Glucose + N(O)	35	5	4.7	-
			10	4.4	1136.4
			15	4.1	-
		45	5	4.3	-
			10	4.3	1142.7
			15	4.5	256.41
		55	5	4.9	1144.7
			10	4.8	946.7
			15	4.7	946.7
2.	Czepak dox +Glucose + N(IO)	35	5	4.1	21.16
			10	3.3	11.13
			15	3.3	10.21
		45	5	4.1	-
			10	4.1	11.11
			15	4.3	-
		55	5	4.5	256.4
			10	4.3	14.4
			15	4.3	12.63
3.	Czepak dox+Sucrose+ N(O)	35	5	3.9	-
			10	3.6	-
			15	3.3	-
		45	5	3.9	-
			10	3.2	14.73
			15	3.6	-
		55	5	4.6	1142.7
			10	4.3	693.6
			15	4.2	596.1
4.	Czepak dox+Sucrose+ N(IO)	35	5	3.6	20.37
			10	3.3	15.91
			15	3.5	16.71
		45	5	3.4	21.42-
			10	3.7	-
			15	3.9	-
		55	5	4.3	8.92
			10	4.5	8.68
			15	3.9	6.56



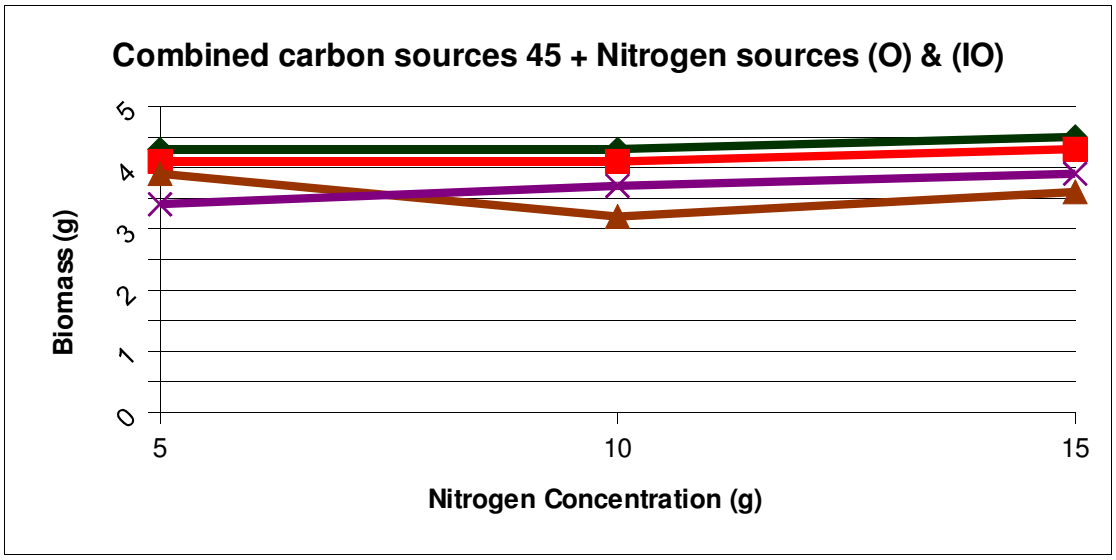
Graph: 1

Czepak dox + Glouse + N (O)

Czepak dox+ Glouse + N (IO)

Czepak dox+ Sucrose + N (O)

Czepak dox+ Sucrose + N (IO)



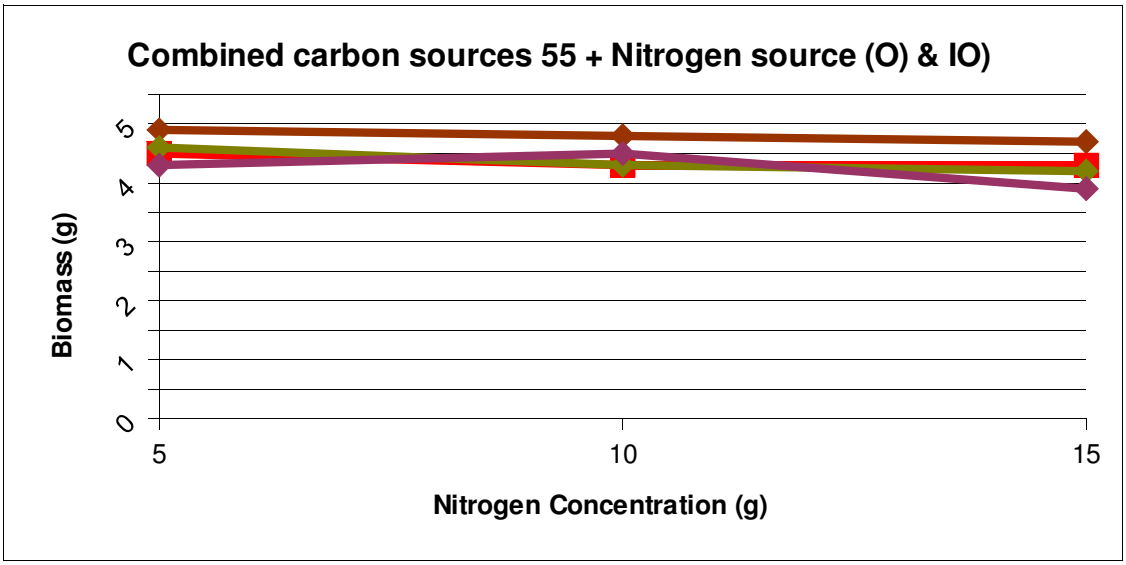
Graph: 2

Czepak dox + Glouse + N (O)

Czepak dox+ Glouse + N (IO)

Czepak dox+ Sucrose + N (O)

Czepak dox+ Sucrose + N (IO)



Graph: 3

Czepak dox + Glouse + N (O)

Czepak dox+ Glouse + N (IO)

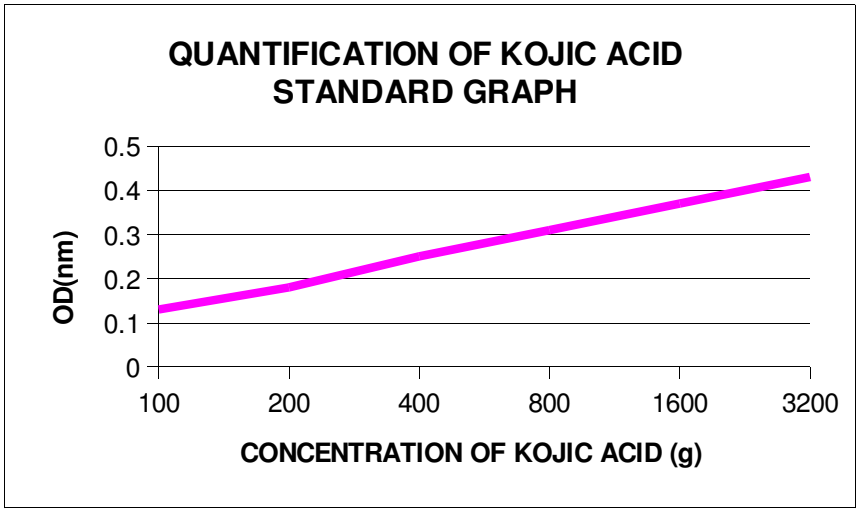
Czepak dox+ Sucrose + N (O)

Czepak dox+ Sucrose + N (IO)

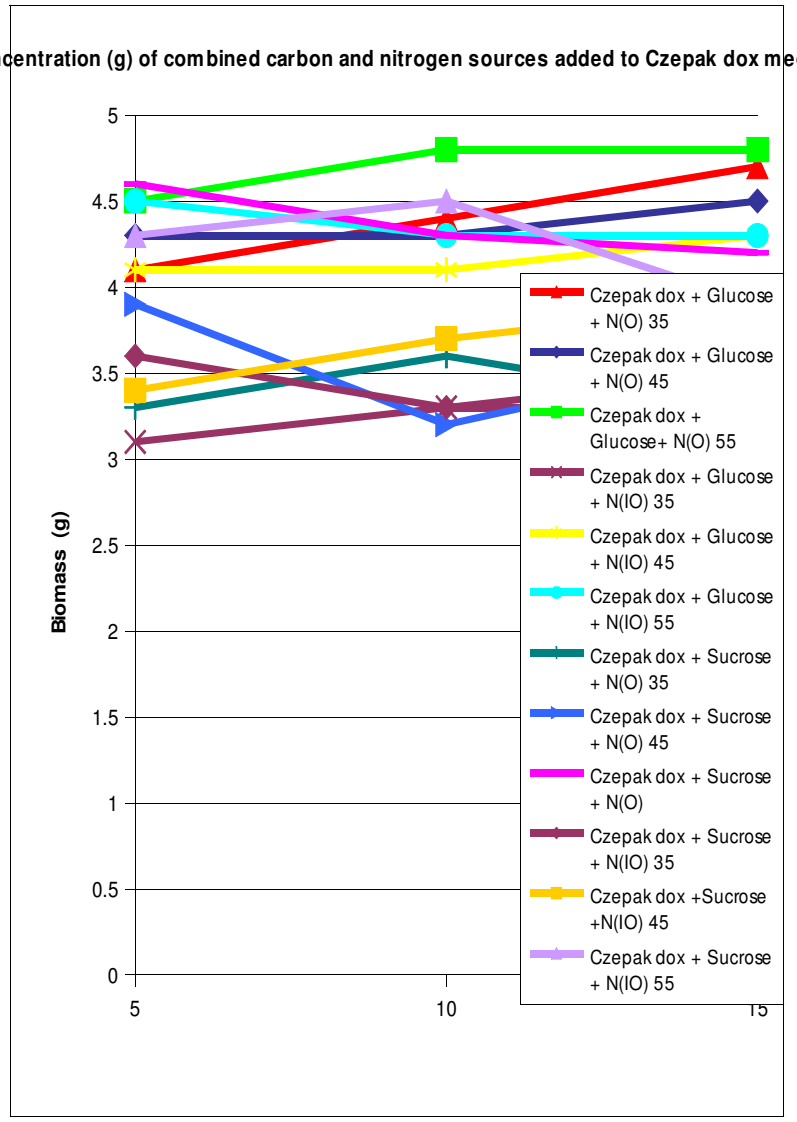
7.1.3 Quantification of Kojic acid

Table 9: Kojic acid quantification (Standard curve)

S. No.	Volume of Kojic acid(µ)	Absorbance (505nm)
1.	100	0.13
2.	200	0.18
3.	300	0.25
4.	400	0.34
5.	500	0.37
6.	600	0.43



Graph 4



Graph 5

7.2 BIOLOGICAL ACTIVITIES OF THE ISOLATED KOJIC ACID

In vitro antimicrobial activity

The biological activity of each compound was investigated against a broad spectrum of microorganisms for antimicrobial activity. Recently Kojic acid isolated from Trichoderma species exhibits potent antimicrobial activity (Rashad M et al 2011). In view of this current study is aimed at evaluating the antimicrobial efficacy of Kojic acid. The results (Table no: 10) showed moderate antibacterial activity against gram-positive bacteria *B. subtilis* and *S. aureus* with a zone of inhibition 10 and 11mm respectively at a concentration of 100 µg/ml and a zone of inhibition 11 and 12mm respectively at a concentration of 200 µg/ml . Gram-negative bacteria was highly sensitive to kojic acid showing a zone of inhibition diameter of 10mm and 16 against *p.vulgaris* and *P. aeruginosa* respectively at aconcentration of 100 µg/ml and a zone of inhibition of 11mm and 18 mm at a concentration of 200 µg/ml.

Antifungal activity of kojic acid was tested against one selected filamentous (*A.niger*) and one unicellular fungi (*c.albicans*) using Zone of inhibition method. Kojic acid affected *A. niger* with a higher zone of inhibition 14mm and 16mm at 100µ g/ml and 200µg/ml concentration respectively. Kojic acid was less potent against other one unicellular fungi *C. albicans* with zone of inhibition diameter of 11mm and 14mm at a concentration of 100µg/ml and 200µg/ml respectively. fluconazole is used as a control.

Table 10 : Susceptability analysis of bacteria and fungi

Susceptability analysis **Zone of inhibition (mm)**					
S.NO.	**STRAIN**	**KOJIC ACID**		**STANDARD**	**STANDARD**
		100µg	200µg	**1**	**2**
1.	*S.aureus*	14	16	25	-
2.	*B.subtilus*	10	11	14	-
3.	*P.vulgaris*	10	12	25	-
4.	*P.aeruginosa*	16	18	23	-
5.	*A.niger*	24	26	-	10
6.	*C.albicans*	13	16	-	36

STANDARD 1 : Penicillin G 10µg

STANDARD 2 : Fluconazole 10µg

Susceptability analysis of bacteria and fungi

Zone of inhibition (mm)

Photo: 1 Sensitivity assay

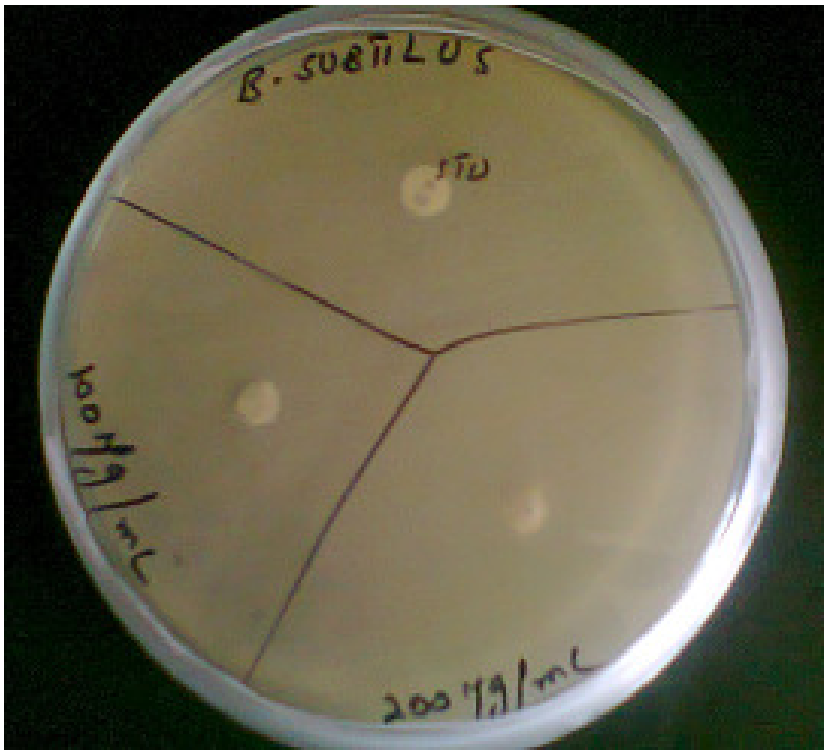


Photo: 2 Sensitivity assay

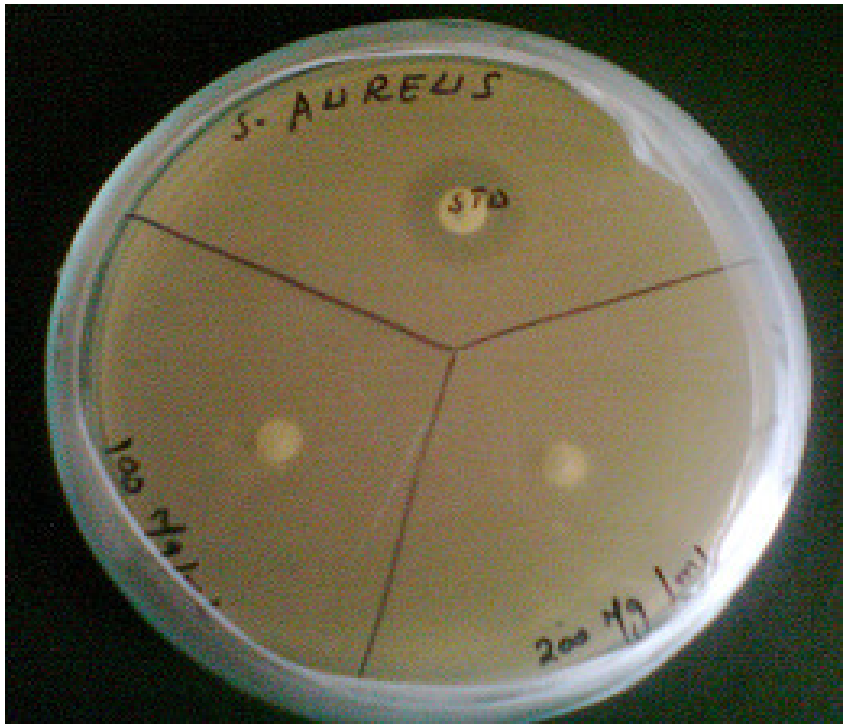


Photo: 3 Sensitivity assay

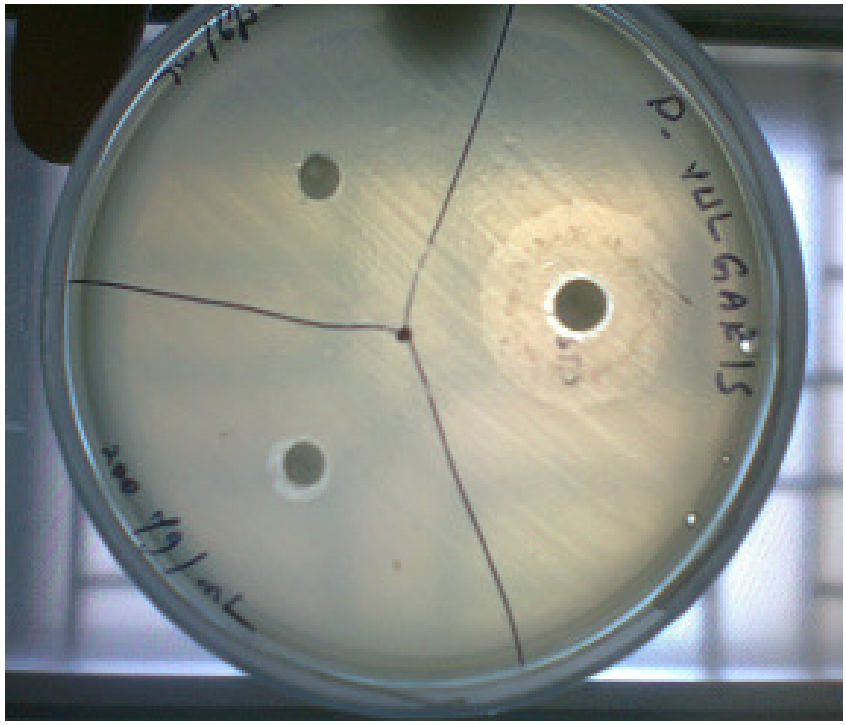


Photo: 4 Sensitivity assay

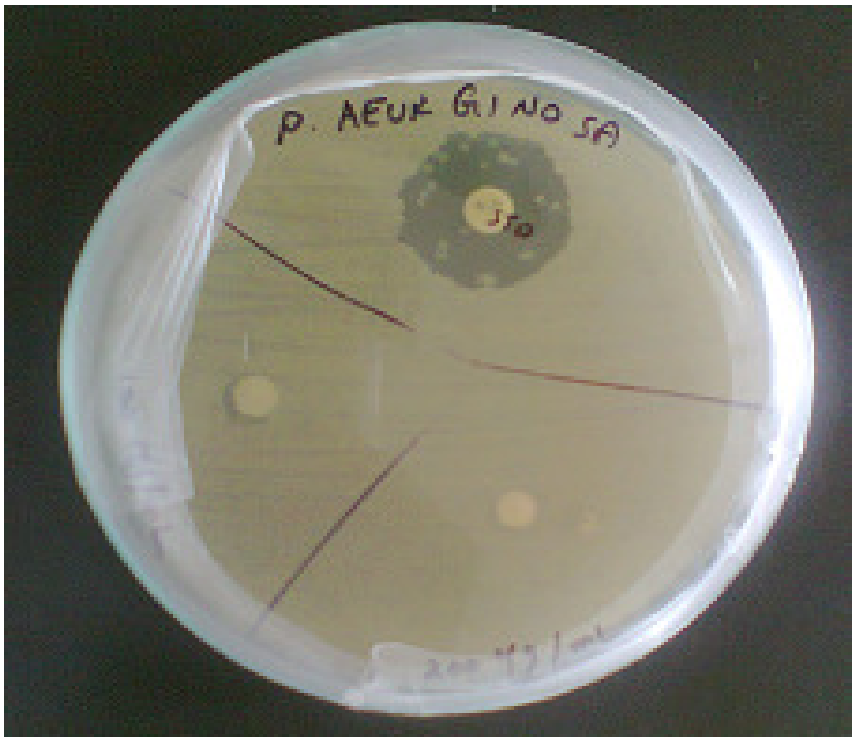


Photo: 5 Sensitivity assay

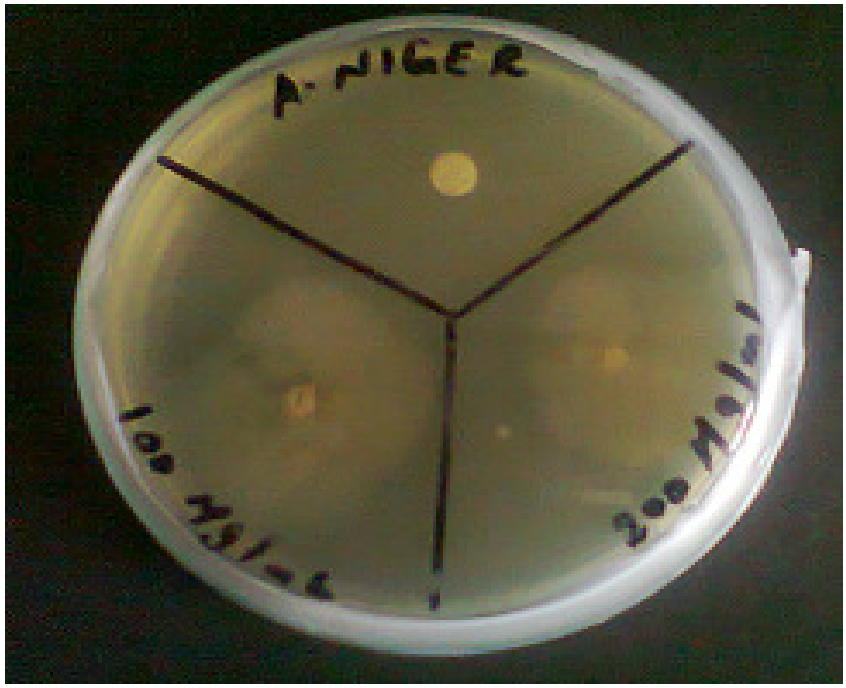


Photo: 6 Sensitivity assay

7.3 Cytotoxicity of Kojic acid

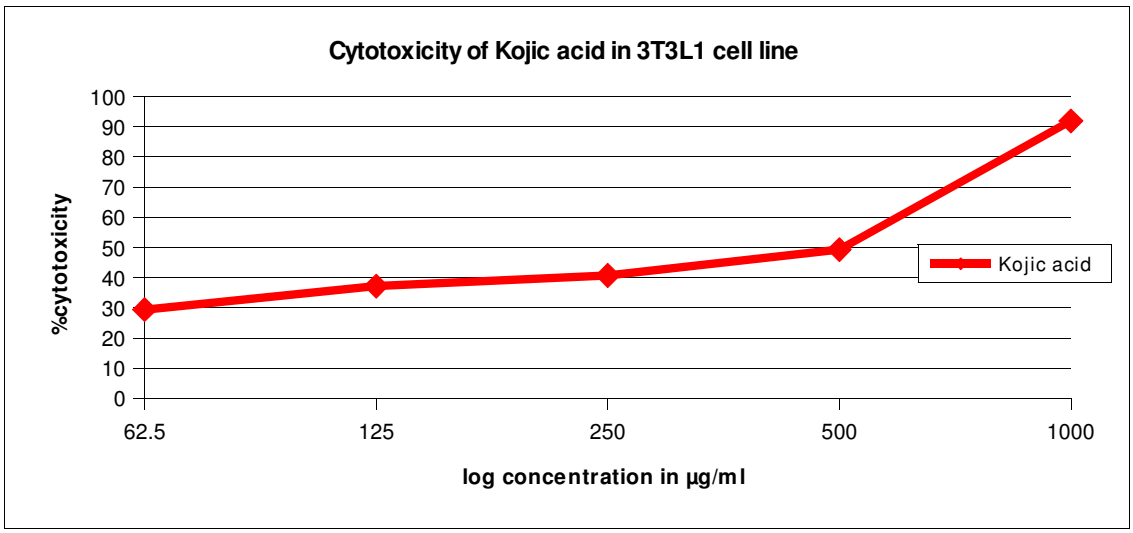
The preliminary objective of the study is to determine the toxicity of kojic acid. The method chosen was MTT cell cytotoxicity assay. In this method the absorbance of the formed formazan crystals were estimated calorimetrically by which the live cells after the treatement with the drug can be estimated. Through this IC_{50} of the drug is calculated using slope established. Here in this study the IC_{50} was found to be 513.33±5.77 µg/ml when tested against 3T3L1 skin cancer cell line (table 11). Many genotoxicity analysis were performed for kojic acid and was reported to have gene toxicity (Satomi Kawaghuchi et al 2007). Hence in this study we may able to report this kojic acid as toxic substance in higher concentrations, from our experimental data.

7.3.1 CYTOTOXICITY EVALUATION

Table 11: Cytotoxic properties of test drugs against 3T3L1 cell line.

S. No	Name of Test sample	Test Concn. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1.	Kojic acid	1000	91.86±0.13	513.33±5.77
		500	49.27±0.27	
		250	40.71±0.88	
		125	37.21±1.44	
		62.5	29.45±0.94	

Graph 6: Graphical representation of cytotoxic effect of Kojic acid



7.4 FT-IR spectrum of Kojic acid

Spectral analysis was carried out to determine the Kojic acid extracted from *Aspergillus niger* MTCC 2208 broth. The IR spectrum of standard Kojic acid and sample obtained after fermentation were compared. The group assigned (Table no 12) in both the spectrum showed strong similarity by which the biosynthesized Kojic acid is confirmed.

Table 12: Interpretation of IR spectrum

S. No.	WAVE NUMBER cm-1 STANDARD	WAVE NUMBER cm-1 SAMPLE	GROUP ASSIGNMENT
1.	3530.8	3632.2	OH STRETCHING
2.	3174	3100	CH STRETCHING
3.	2924.19	2828.09	CH STRETCHING
4.	1702.86	1661.07	C=O STRETCHING
5.	1579.43	1611.85	C=C STRETCHING
6.	1282	1202	C-O STRETCHING
7.	941	847	C-H DEFORMATION

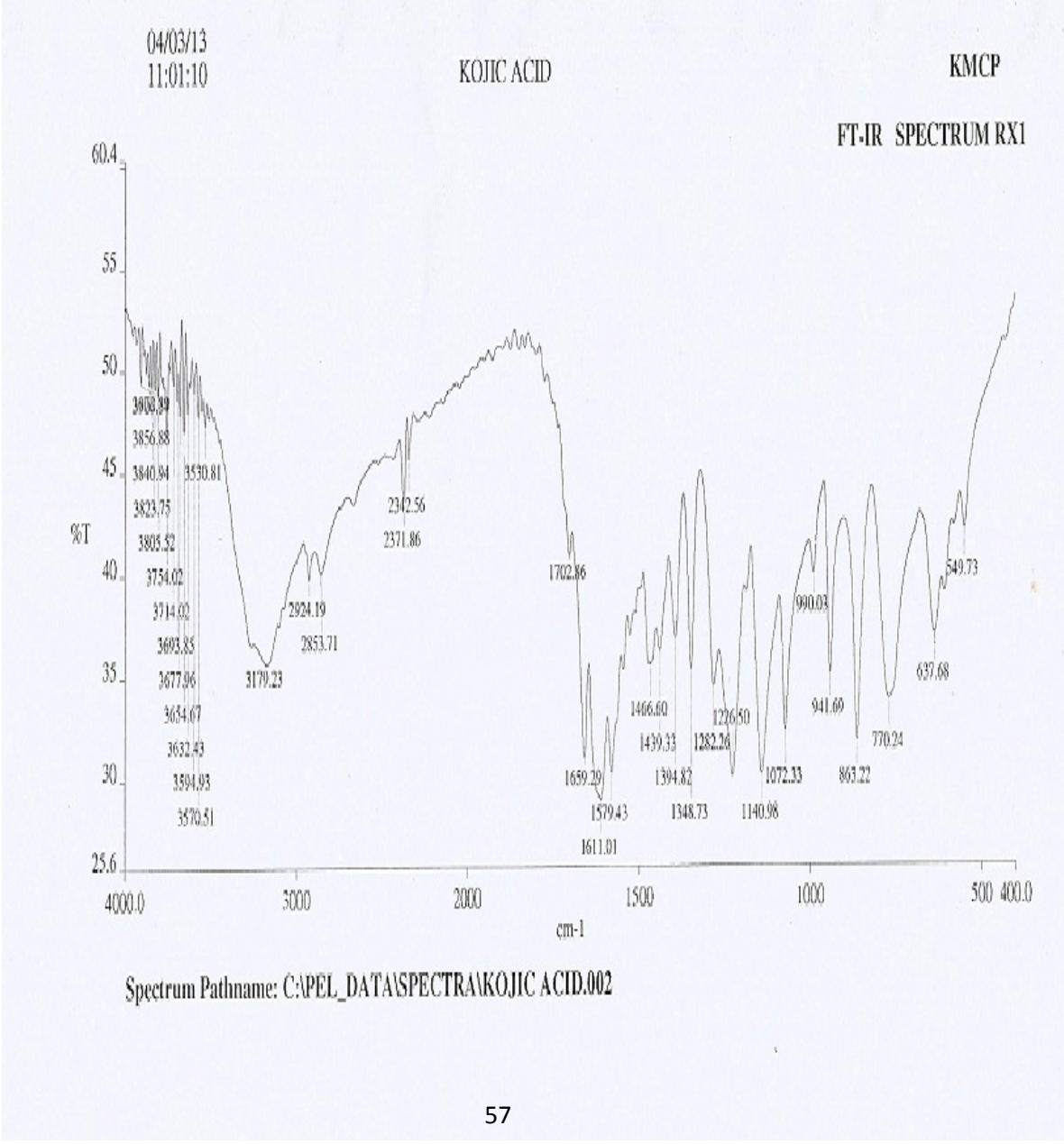
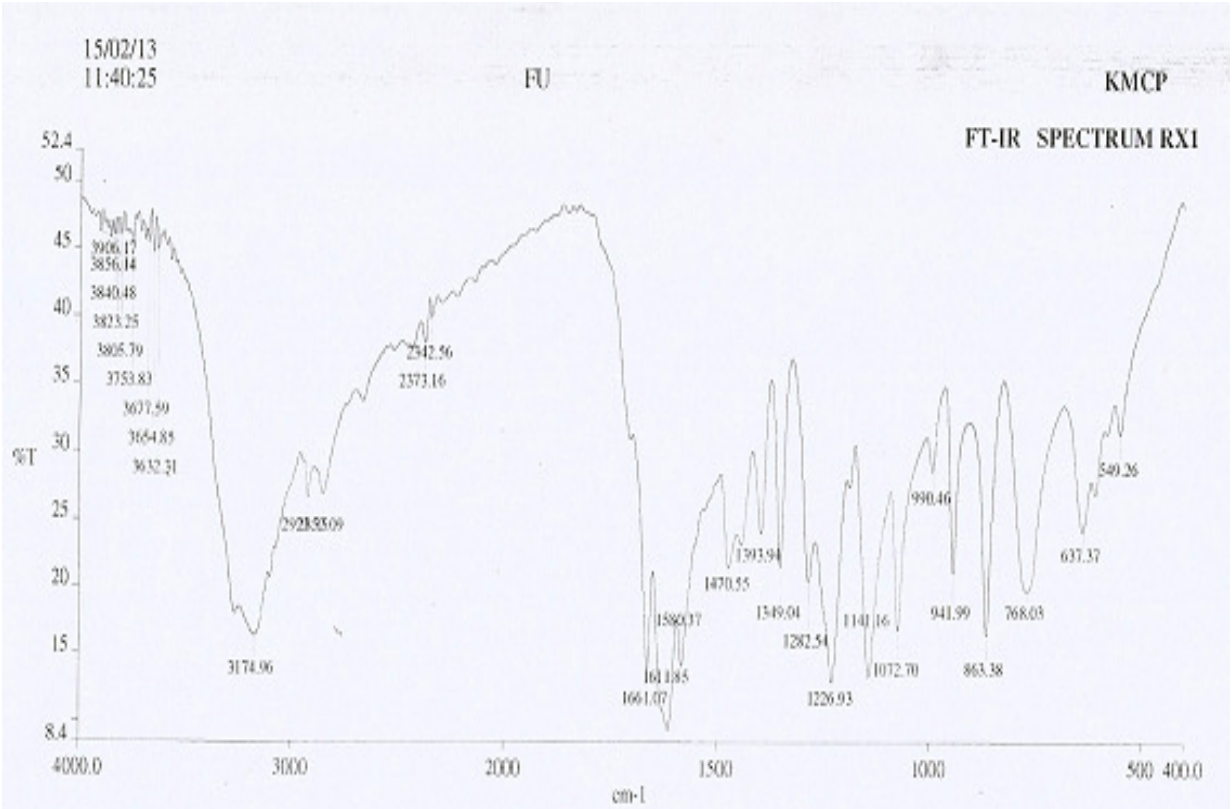

04/03/13
11:01:10
KOJIC ACID
KMCP
FT-IR SPECTRUM RX1
%T
60.4
55
50
45
40
35
30
25.6
4000.0
3000
2000
1500
1000
500
400.0
cm-1
3856.88
3840.94
3530.81
3823.75
3805.52
3754.02
3714.02
3693.85
3677.96
3634.67
3632.43
3594.93
3570.51
3179.23
2924.19
2853.71
2342.56
2371.86
1702.86
1659.29
1611.01
1579.43
1466.60
1439.33
1394.82
1348.73
1282.26
1226.50
1140.98
1072.33
990.03
941.69
863.22
770.24
637.68
549.73
Spectrum Pathname: C:\PEL_DATA\SPECTRA\KOJIC ACID.002

Figure 2: IR spectrum of Standard Kojic acid

-



Spectrum Pathname: C:\pel_data\spectra\FUN.002

FUN.pk

FUN.002 3601 4000.00 400.00 9.29 48.69 4.00 %T 4 1.00

REF 4000 48.69 2000 44.70 600
3906.17 46.19 3856.14 45.78 3840.48 45.96 3823.25 45.99 3805.79 46.01
3753.83 45.31 3677.59 45.33 3654.85 44.66 3632.31 44.88 3174.96 16.31
2923.35 26.57 2852.09 26.83 2373.16 37.96 2342.56 39.85 1661.07 13.81
1611.85 9.29 1580.37 14.04 1470.55 21.42 1393.94 23.93 1349.04 21.50
1282.54 20.36 1226.93 12.89 1141.16 13.24 1072.70 16.74 990.46 28.56

Figure 3: IR spectrum of Isolated Kojic acid

8. SUMMARY AND CONCLUSION

The selected fungal strain for our study is *Aspergillus niger* MTCC 2208 and was procured from IMTECH, Chandigarh and maintained in CYA slant, the fermentation process (submerged) was performed by adding 5 ml of *Aspergillus niger* MTCC 2208 inoculum culture in sterile Modified Czapek- Dox medium and was incubated at 25 ± 2^{o} C for 14 days.

Submerged fermentation technique was adapted to biosynthesize Kojic acid from *Aspergillus niger.* The highest production of kojic acid was obtained at a concentration of 55g glucose and 5g of peptone as an organic a nitrogen source and the yield was 1144.7µg, from this study we concluded that the production of Kojic acid increased with the increase of carbon source such as glucose and decreased with the decrease of organic nitrogen source such as peptone, the Kojic acid which was obtained from the 49 runs were pooled together and utilized to evaluate its antimicrobial and cytotoxic activity.

Kojic acid was tested against four bacterial strains and two fungal strains using Disc diffusion method. Kojic acid showed moderate antibacterial activity when compared with the antifungal activity which gave good potency. Kojic acid is tested against Skin cell lines (3T3L1) and IC_{50} was found to be 513.33±5.77 µg/ml.

By all these results we can conclude that biosynthesize of kojic acid need excess concentration of carbohydrate source with decreased amount of nitrogen organic source. Kojic acid definitely possesses antifungal activity with high potency rather than antibacterial activity. From the cytotoxic report it can be concluded that kojic acid upon high concentration produce toxic to the skin.

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